Sanguinarine: Some analytical, pharmacological and synthetic studies.

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1978

Awarding institution:
University of Bath

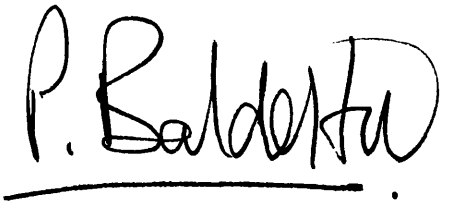
SANGUINARINE:
SOME ANALYTICAL, PHARMACOLOGICAL AND SYNTHETIC STUDIES

Submitted by Peter Balderstone

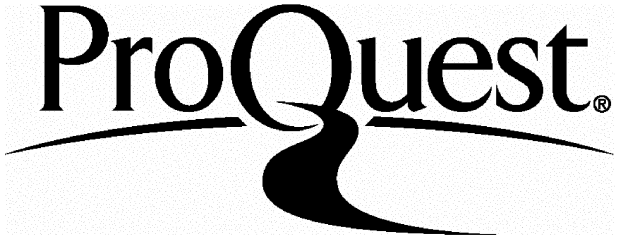
for the degree of Doctor of Philosophy

of the University of Bath

1978



P BALDERSTONE



To Hilary, my wife

ACKNOWLEDGEMENTS

I should like to thank my supervisor, Dr S F Dyke, for his help and interest during the course of my research. I should like to thank Dr A J Floyd for his supervision during the absence of Dr Dyke on sabbatical leave, and Dr R G Kinsman for his help, friendship and support during my stay in Bath.

The Medical Research Council is gratefully acknowledged for its financial support.

The help and advice of fellow postgraduates and members of the staff, academic and non-academic, is gratefully acknowledged, as is the support given by my wife during my research and in the preparation of this thesis.

Finally, I should like to draw the attention of the reader to the enthusiasm and dedication of the late Dr S A E Hakim of Bombay, whose work stimulated the research described herein.

Dr Hakim died of cancer of the stomach at the Royal Free Hospital, London, in 1976.

CONTENTS

PREFACE

The work described in this thesis was carried out in the School of Chemistry of the University of Bath between October 1972 and September 1975.

Mass Spectra were recorded on an AEI MS12 mass spectrometer.

NMR Spectra were obtained with either a Varian A60 (60 MHz) or a Jeol 100 MHz spectrometer.

IR Spectra were obtained with a Perkin Elmer PE237 spectrophotometer.

UV Spectra were obtained with a Perkin Elmer PE402 spectrophotometer.

Gas Chromatography was carried out using a Pye 104 Gas Chromatograph.

SUMMARY

The work described in this thesis was stimulated by the observations of several Indian workers who had endeavoured to show a link between the ingestion of products of the poppy Argemone mexicana, Linn. and the development of a variety of debilitating diseases, notably the eye condition Glaucoma, although other complaints, mainly cardiac and circulatory were also noted. The role of sanguinarine as an environmental carcinogen was also suggested.

Chapter 1 of this thesis comprises a summary of the occurrence, biosynthesis, and in-vitro synthesis of benzo(c)phenanthridine alkaloids in general and sanguinarine, 2,3,7,8-dimethylenedioxy-5-methyl-benzo(c)phenanthridine, in particular.

Chapter 2 attempts to summarise the pharmacological and toxicological properties of sanguinarine and of the papaver Argemone mexicana, Linn. in the context of the aetiology of the eye disease glaucoma. A quantitative analytical technique for detecting sanguinarine in physiological quantities is described and examination of material from clinical cases of Argemone poisoning is reported.

Chapter 3 discusses the possible role of sanguinarine or a metabolite as an environmental carcinogen. The results of feeding experiments and of the examination of tissue samples

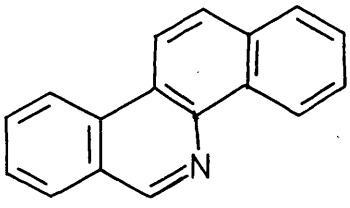
are reported.

Chapter 4 describes some synthetic studies undertaken to explore routes to specifically radio-labelled sanguinarine derivatives for use in the investigation of the postulated *in-vivo* rearrangement of sanguinarine.

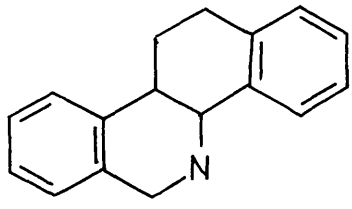
CHAPTER 1

OCCURRENCE

Up to the present time, twenty-three naturally occurring benzo(c)phenanthridine alkaloids have been characterised in the literature. Of these, twelve are derived from the unsaturated skeleton (1) and are shown in Table 1. A further group of ten are based on the unsaturated skeleton (2): These are shown in Table 2.

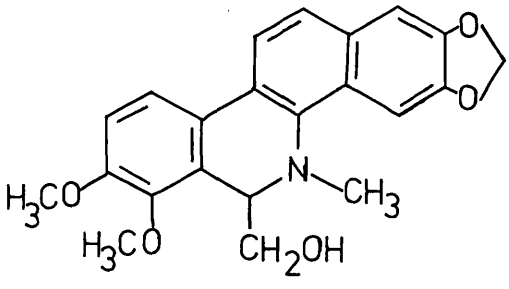


(1)



(2)

Finally, one alkaloid, bocconoline (3) has been shown to be based on the 5,6-dihydrobenzo(c)phenanthridine skeleton (4).



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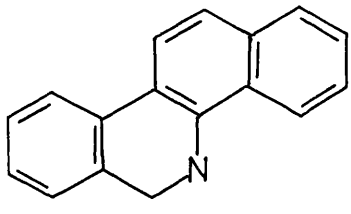
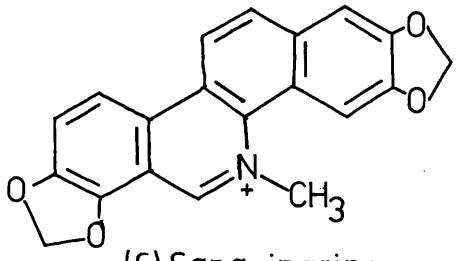


(4)

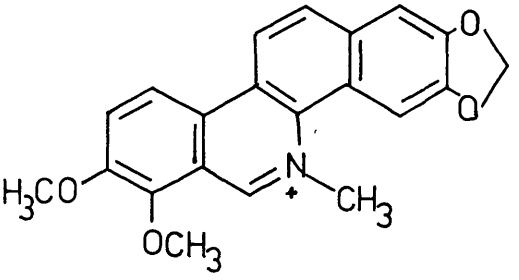
Several artefacts have been isolated and these are shown in Table 5. It can be seen that the majority of these artefacts

TABLE 1

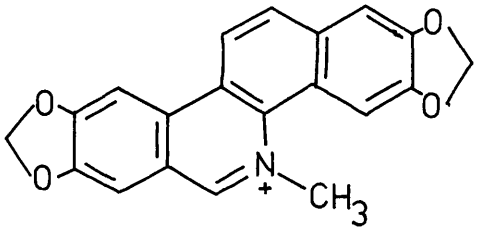
Fully unsaturated alkaloids



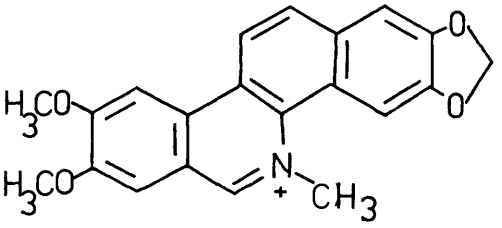
(6) Sanguinarine



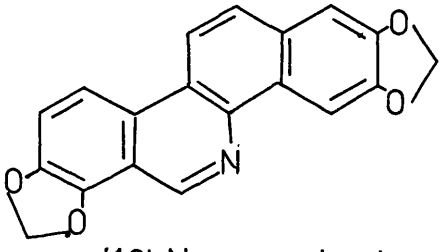
(7) Chelerythrine



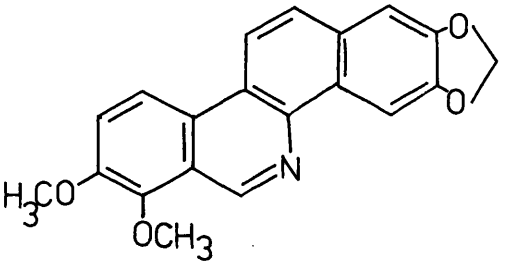
(8) Avicine



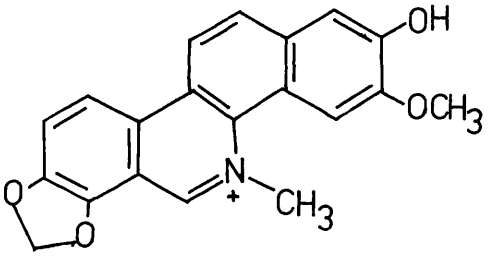
(9) Nitidine



(10) Norsanguinarine



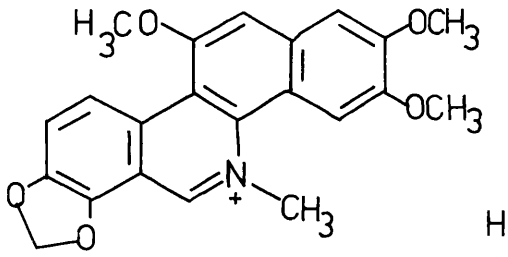
(11) Norchelerythrine



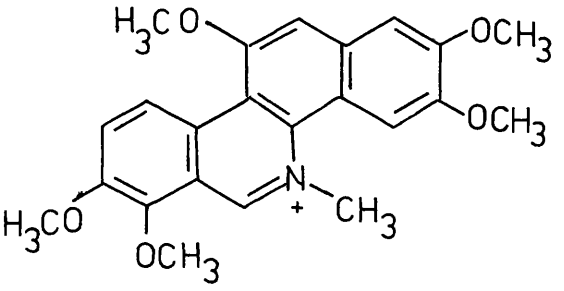
(12) Fagaronine

TABLE 2

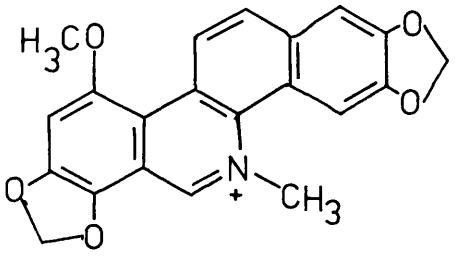
Fully unsaturated alkaloids with more than four oxygen atoms



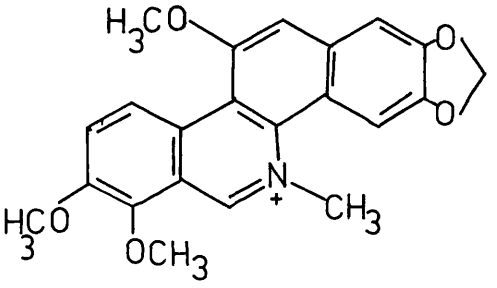
(13) Sanguirubine



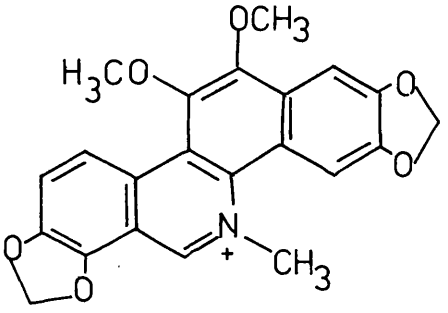
(14) Sanguilutine



(17) Chelirubine



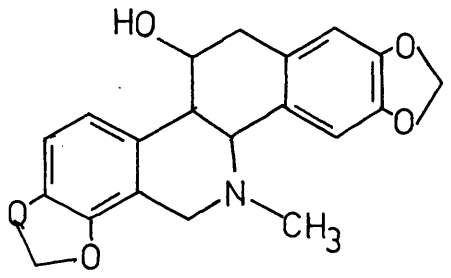
(16) Chelilutine



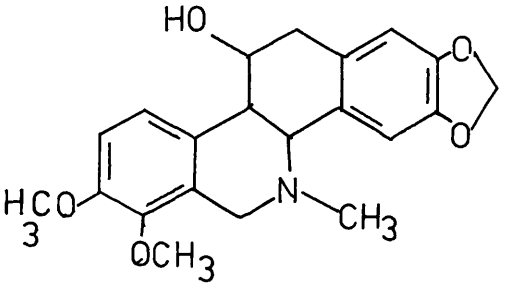
(19) Macarpine

TABLE 3

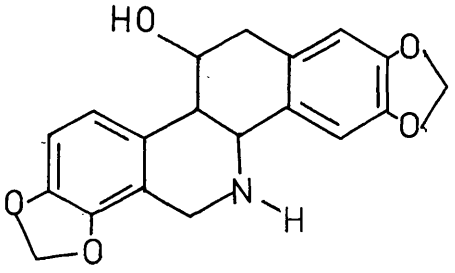
Partially unsaturated alkaloids



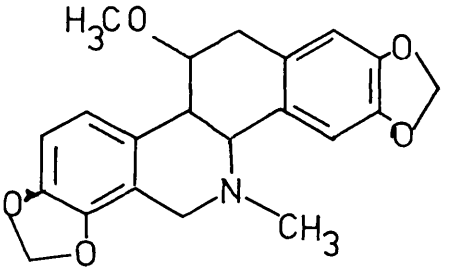
(20) Chelidonine



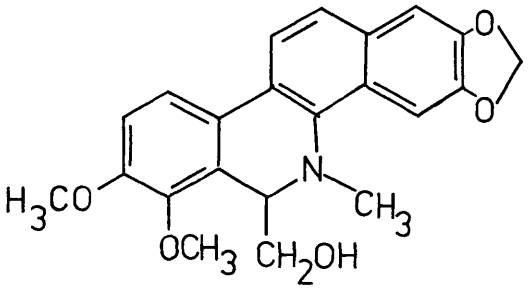
(21) Homochelidonine



(22) Norchelidonine



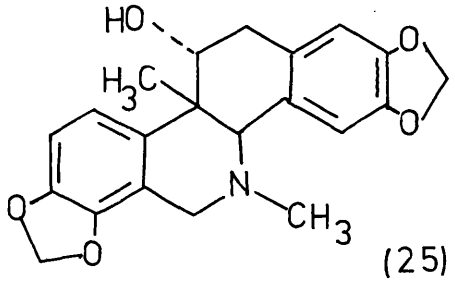
(23) Methoxychelidonine



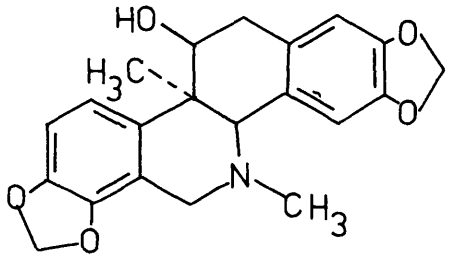
(24) Bocconoline

TABLE 4

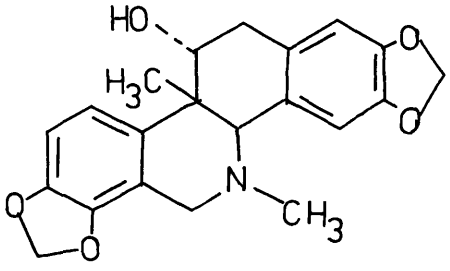
Partially unsaturated alkaloids with methyl substituent at C-13



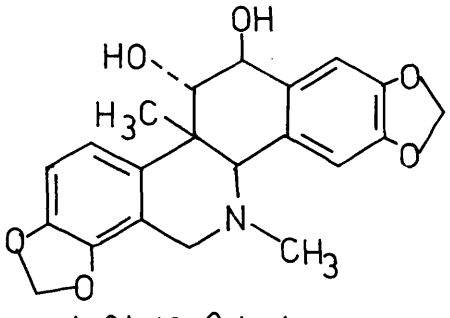
(25) Corynoline



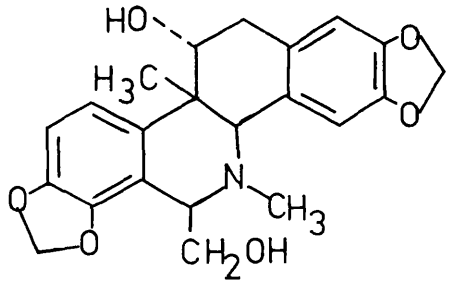
(26) 14-epicorynoline



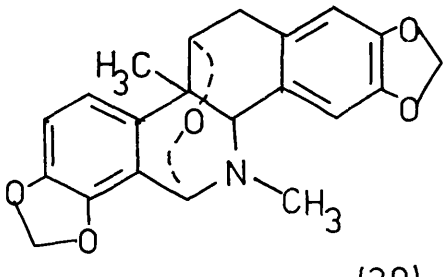
(27) 11-epicorynoline



(28) 12-β-hydroxycorynoline



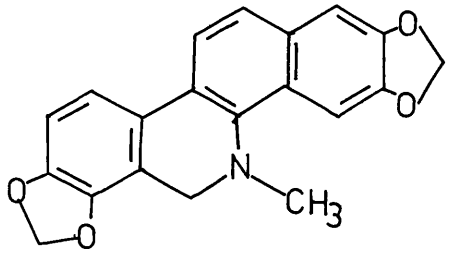
(29) Corynolamine



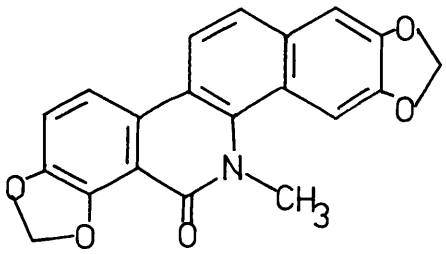
(30) Corynoloxine

TABLE 5

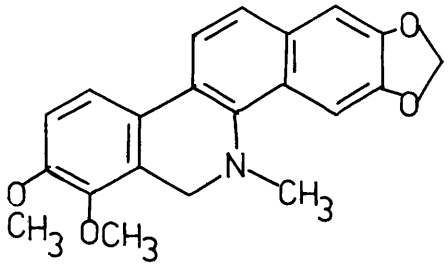
Artefacts



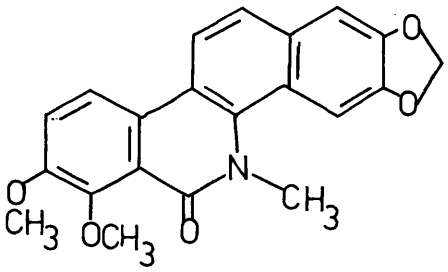
(31) Dihydrosanguinarine



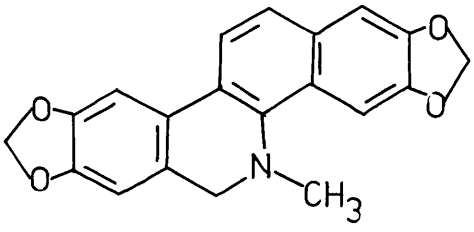
(32) Oxysanguinarine



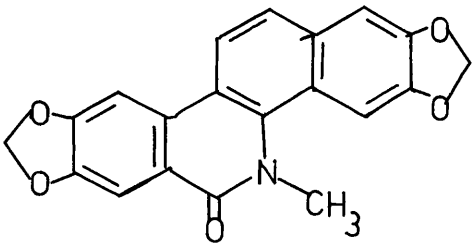
(33) Dihydrochelerythrine



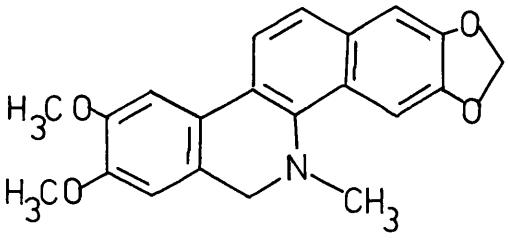
(34) Oxychelerythrine



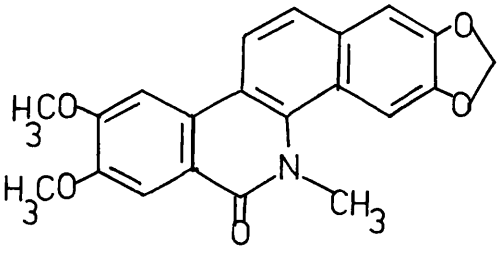
(35) Dihydroavicine



(36) Oxyavicine

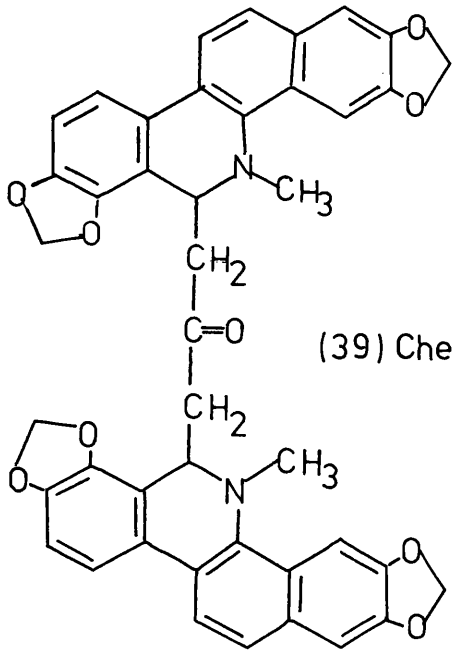


(37) Dihydronitidine

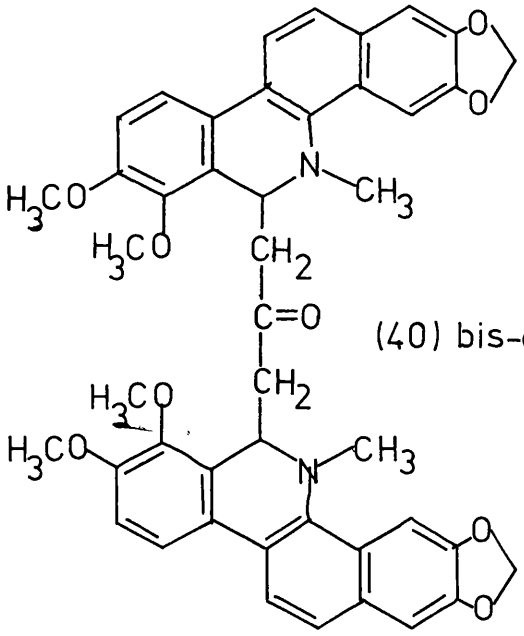


(38) Oxynitidine

TABLE 5 cont'd

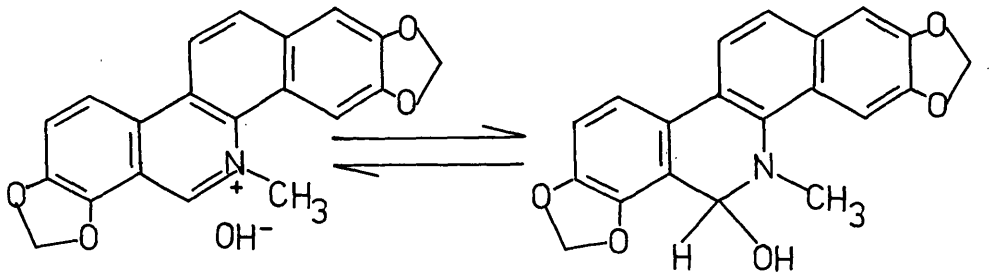


(39) Chelidimerine



(40) bis-chelerythrinyl acetone

arise from disproportionation of the pseudobase (5). Thus,



(6) (5)

sanguinarine (6) gives rise to dihydrosanguinarine (25) and oxysanguinarine (26). The disproportionation is thought to take place during the isolation procedure and that it is not an in-vivo process.

The majority of the work on this class of compounds has been carried out in the last twenty years although sanguinarine (6) was first isolated[1] in 1829. Prior to 1959, five alkaloids had been isolated: sanguinarine (6), chelerythrine (7), chelidonine (20), homochelidonine (21) and methoxychelidonine (23).

Avicine (8) andnitidine (9) were identified by Arthur and Ng[2] in 1959. The methoxylated derivatives chelirubine (15), chelilutine (16), sanguirubine (13), sanguilutine (14) and marcapine (19) were originally identified by Slavik[3] and were thought to be methoxylated in Ring B. More recent work by Ishii et al[4] has characterised chelirubine as structure (17) and

shows its identity with bocconine, previously thought to be structure (18). This has cast doubt on the original characterisations, and the structures and biogenesis of the other four alkaloids is now ambiguous.

Three nor-N-methyl compounds occur: norchelidonine (22) and norsanguinarine (10) were discovered by Slavik[6,7], while norchelerythrine (11) was discovered by Govindachari and Viswanathan[8].

The two 13-methylbenzo(c)phenanthridines corynoline (25) and corynoloxine were both first characterised by Takao[9,10], whose group[11] also isolated the two epimers 14-epicorynoline (26) and 11-epicorynoline (27). Two other 13-methyl derivatives have been characterised: 12-B-hydroxycorynoline (28) by Nonaka and Nishioka[12] and corynolamine (29) by Ishii, Hosoya and Takao[13].

Bocconoline (24) was discovered by Ishii, Hosoya and Takao[14].

Fagaronine (12) with a phenolic function in ring D was first isolated by Messmer et al[15] and subsequently synthesised by Stermitz and his group[16].

DISTRIBUTION

Benzo(c)phenanthridines, like other isoquinoline alkaloids, are found in the two botanical Orders RHOEDALES and RUTALES.[17] Within these orders, and again following the pattern of isoquinoline alkaloids in general, benzo(c)phenanthridine alkaloids are found in the Families PAPAVERACEAE and FUMARIACEAE of the Order Rhoedales and in the Family RUTACEAE of the Order Rutales. Much of the chemotaxonomic work has been carried out by the groups of Slavik and of Stermitz.

Originally, benzo(c)phenanthridine alkaloids were believed to be very rare, but it is now becoming apparent that members of this class of alkaloids, particularly sanguinarine (6), although present usually in trace quantities, are extremely widely distributed.[18] Indeed, work by Hakim, Mijovic and Walker, who screened 53 species of papaveraceae and fumariaceae, showed sanguinarine (6) to be present in nearly every case. This led them to postulate the distributions of sanguinarine throughout a large proportion of all the 675 species of papaver and fumaria.

The most prolific source of benzo(c)phenanthridine alkaloids are the papavers Sanguinaria canadensis[17] and Chelidonium majus[18], whilst the plant in which sanguinarine

appears to be most abundant is *Argemone mexicana*.[17]

The results of an investigation into the chemotaxonomy of the genus Argemone carried out by Stermitz and his group[20,21] are summarised in Table 6.

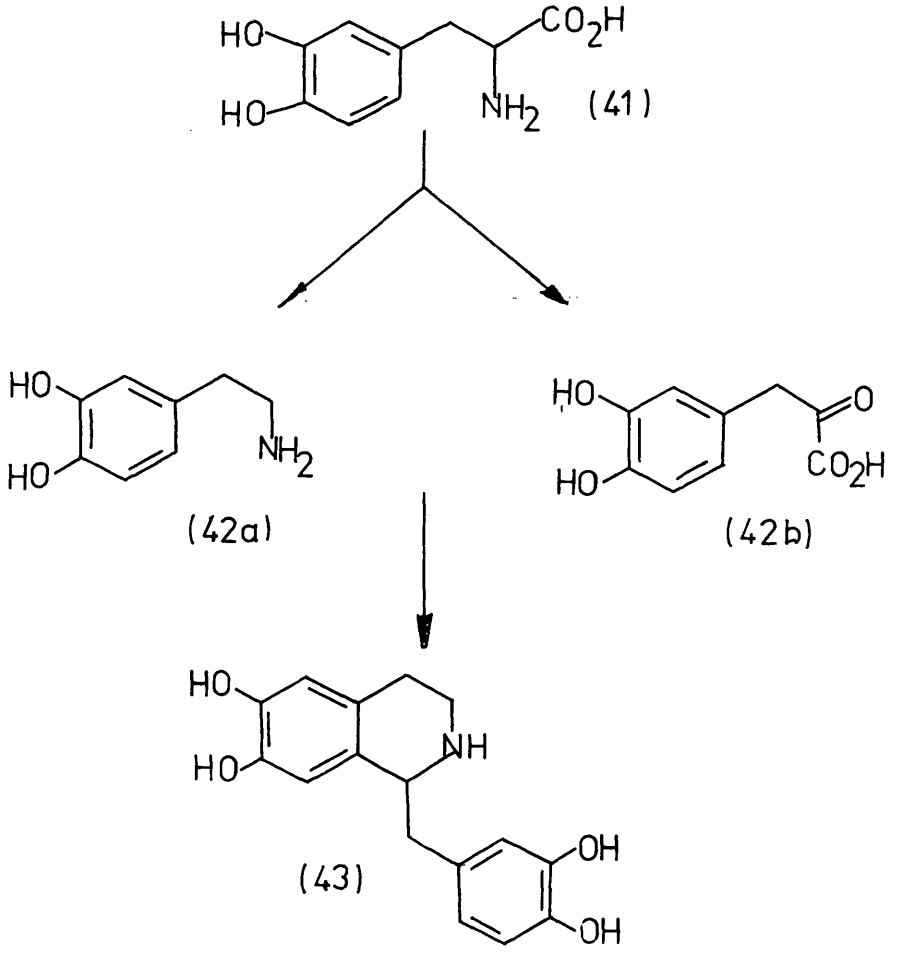
	Sanguinarine	Chelerythrine	Berberine	Protopine	Allocryptopine	Coptisine	Cryptopine	Argemonine	Norargemonine
A. mexicana	x	x	x	x	x	x	x		
A. albiflora	x		x	x	x	x			
A. brevicornita			x						x
A. subfusiformis	x	x	x	x	x				
A. echimata			x				x		
A. glauca	x	x	x	x	x				
A. pleiacantha			x	x	x		x		
A. gracilata				x				x	
A. polyanthemos	x		x	x	x				
A. corymbosa	x		x	x	x				
A. chisosensis			x	x	x				
A. sanguinea			x			x		x	
A. aurantiacei				x			x		
A. grandiflora			x	x	x				

ALKALOIDS OF THE GENUS ARGEMONE

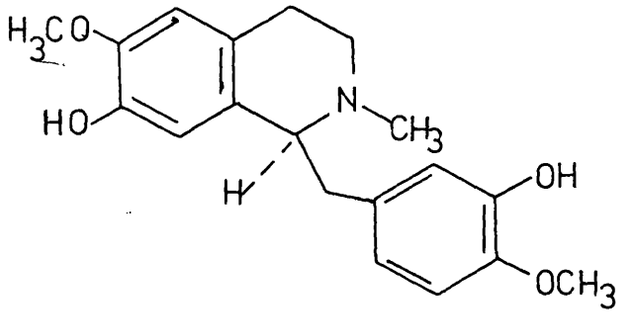
TABLE 6

BIOSYNTHESIS

The biosynthesis of isoquinoline alkaloids in general and benzo(c)phenanthridine alkaloids in particular is now well researched and fairly well understood. It is thought that the key bio-intermediate is a 1-benzylisoquinoline derived via norlaudanosoline (43), from dopamine (42a). Dopamine is thought to be formed by the decarboxylation of dihydroxy-phenylalanine (41). Dopamine condenses with the α-ketoacid (42b) formed by oxidation of dihydroxyphenylalanine to give norlaudanosoline (43).



Experiments[22] have shown that the necessary 1-benzylisoquinoline is (+)-reticuline (44), the partially O-methylated derivative of norlaudanosoline (43).



(44)

Due to enzyme stereospecificity it is considered that only the (+) isomer is utilised in benzo(c)phenanthridine biosynthesis: indeed, benzo(c)phenanthridine biosynthesis is now seen as one of many biosynthetic routes which fall within the general scheme of isoquinoline biosynthesis from the common precursor norlaudanosoline. Thus, norlaudanosoline forms the (+)- and (-)-isomers of reticuline: the (-)-isomer is thought[23] to be the precursor of the morphine group of alkaloids, whereas the (+)-isomer forms the tetrahydroprotoberberines and thus the protoberberines, phthalide isoquinolines and benzo(c)phenanthridines. Another route from norlaudanosoline leads to

orientaline, the precursor of the proaporphines and aporphines.

Benzo(c)phenanthridine biosynthesis has been studied by Battersby, by Leete, by Stermitz and by others and in fairly recent work made possible by the increased sensitivity of modern radio-labelling techniques.

The bulk of the work has concentrated on chelidonine (20) but has been applied *inter alia* to sanguinarine.

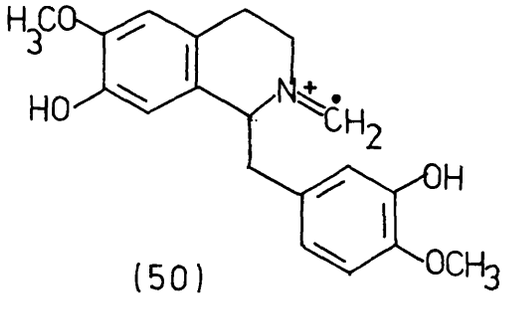
In an experiment by Leete[25], (±)-tyrosine-2-^{14}C (45) was fed to *Chelidonium majus* and chelidonine-11-^{14}C-14-^{14}C (46) was isolated, as was similarly labelled sanguinarine (47). Degradative experiments showed the active chelidonine to be unequally labelled, with 61% of the activity at C-11, indicating the possibility that tyrosine (or more probably its hydroxylated derivative) is converted *in-vivo* to two metabolites which subsequently condense.

A further experiment[26] in which labelled dopamine (48) was fed to *C. majus* led to the isolation of chelidonine labelled at C-11 and also active (±) stylopine-6-^{14}C (49). This led to a final experiment[27] in which the tetrahydroprotoberberine, (±)-stylopine-6-^{14}C was fed to *C. majus* and chelidonine-11-^{14}C recovered, showing that the benzo(c)phenanthridine skeleton is formed by the rearrangement of a tetrahydroprotoberberine

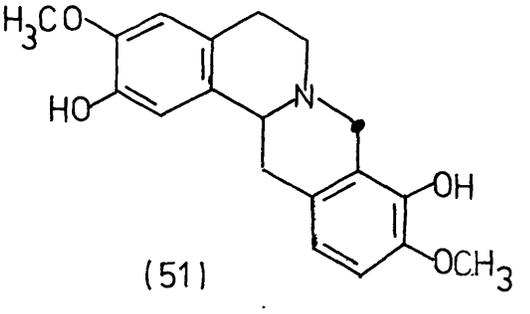
intermediate (cf. references 28,29).

These experiments are summarised in Scheme No. 1.

Battersby[30] and Barton[31] fed (+)-reticuline-2-^{14}C (50) to C.majus. Specifically labelled scoulerine (51) was isolated. Two reaction pathways were postulated, both involving a reaction of the N-oxide: that proposed by Barton proceeding via a rearrangement to the N-hydroxymethyl compound and subsequent condensation, whereas the more likely Battersby route involves oxidation to the imine followed by a Mannich type condensation, viz:



(50)

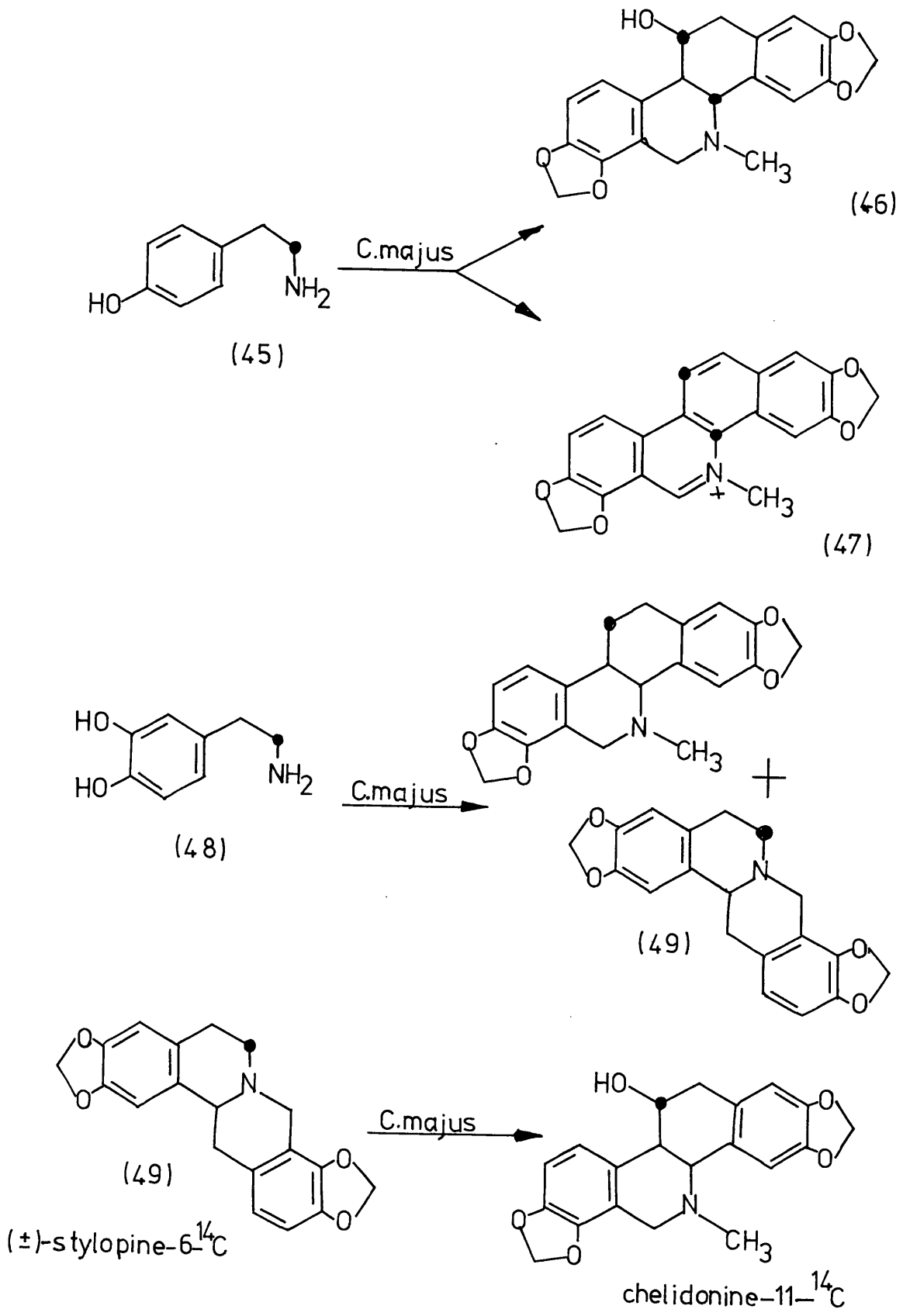


(51)

the specificity of the label was proved by degradation.

Using reticuline-2-^{14}C condensation can take place ortho- to the 1-benzyl hydroxy group to form scoulerine (39) as reported for C. Majus or para- to the same group to form coreximine (52)

SCHEME 1


HO
O
O
N
CH3
O
O
(46)
HO
NH2
C.majus
(45)
O
O
N+
CH3
O
O
(47)
HO
HO
NH2
(48)
C.majus
O
O
N
CH3
O
O
+
O
O
N
O
O
(49)
O
O
N
O
O
(49)
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HO
O
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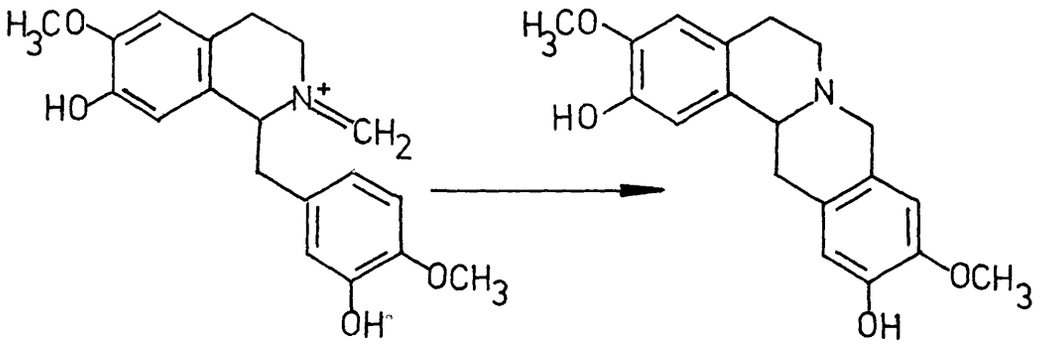
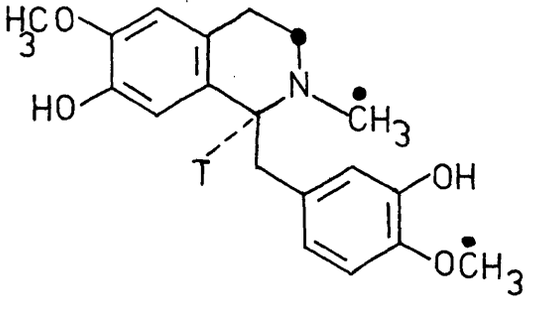
N
CH3
O
O
chelidonine-11-14C



(52) Coreximine

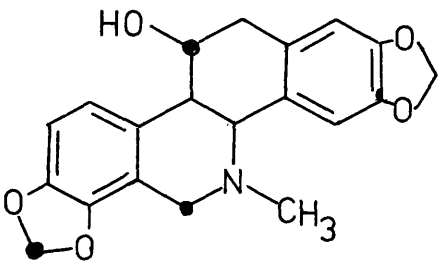
as reported by Manske[32] for Dicentra eximia.

Scoulerine, thus formed can undergo several oxidative processes. These were investigated by Battersby et al. who fed (+)-reticuline labelled (53) with ^{3}H and ^{14}C



(53)

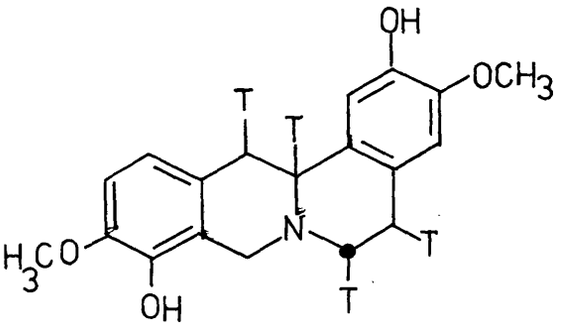
the chelidonine extracted from the plant was found to be labelled only with ^{14}C and, by degradation, in the positions shown (54)



(54)

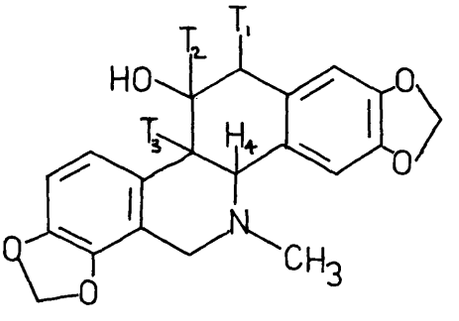
This experiment reinforced the 'berberine bridge' theory and indicated that methylenedioxy groups are formed *in-vivo* from the o-methoxyphenol system (this has since been achieved[33] *in-vitro*), but more importantly the absence of ^{3}H at the benzo(c)phenanthridine C-14 position (and thus its initial complete loss from protoberberine C-14 position) indicated the formation of a 1,2-dihydroisoquinoline intermediate.

In order to investigate this further the tetrahydroprotoberberine scoulerine was prepared[34] with ^{3}H labels at C-5, C-13 and C-14 and a reference ^{14}C label at C-6, viz (55)



(55)

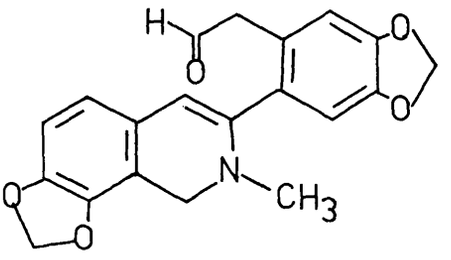
the label positions in the extracted chelidonine were found by degradation:



	%T
T_1	100
T_2	50
T_3	<50
H_4	0

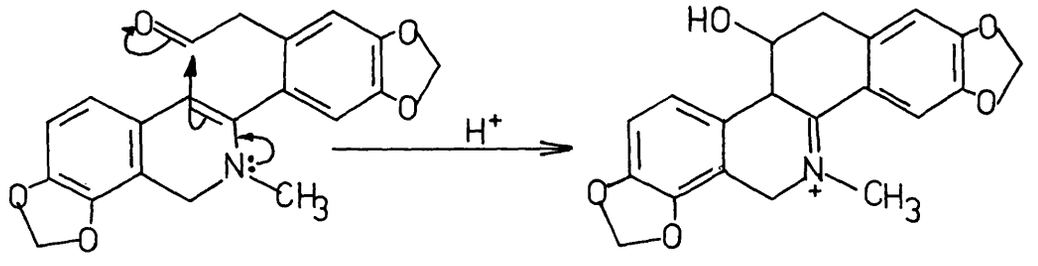
The relative activities were shown to be (referring to scoulerine): 100% loss of ^3H from C-14, no loss from C-5, 50% loss from C-6, less than 50% from C-13. Battersby rationalised the above data by postulating the existence of the 1,2-dihydro-isoquinoline intermediate (56) shown below; the oxidation at C-6 is obviously stereospecific, as is the formation of the

C14–N double bond. The isomerisation to the 1,2-dihydroisoquinoline is non-specific, leading to the observed isotope effect.



(56)

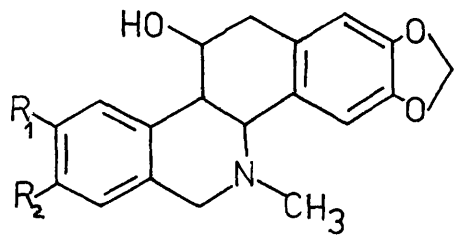
Cyclisation to the chelidonine type of compound is straightforward:



FULLY UNSATURATED DERIVATIVES

It is thought that the fully aromatic benzo(c)phenanthridinium salts are derived from the corresponding chelidonine type of compound by a process involving dehydration and oxidation. Thus sanguinarine(6) and chelerythrine(7) are derived from scoulerine(51) via chelidonine(20)and homochelidonine(21). Similarly avicine(8) and nitidine(9) are derived from coreximine(52).

It is tempting to accept this; certainly, sanguinarine and chelerythrine often occur together with chelidonine and homochelidonine and the conversion of, for example, chelidonine into sanguinarine is a simple process <u>in-vitro</u>. It should be pointed out, however, that there is no confirmatory evidence and that the 11-hydroxy compounds (57a) and (57b),



(57)

	R_1	R_2
(57a)	$-O-CH_2-O-$	
(57b)	$-OCH_3$	$-OCH_3$

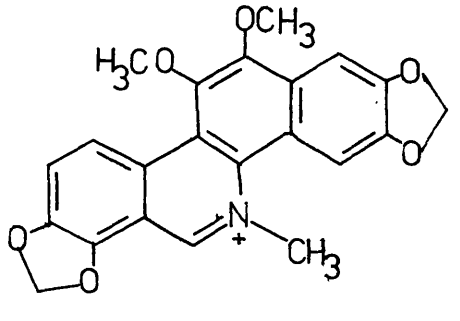
which would lead to avicine (57a) and nitidine (57b) have yet to

be found in Nature. It is possible then that sanguinarine is produced by a separate (although parallel) route to that which produces chelidonine.

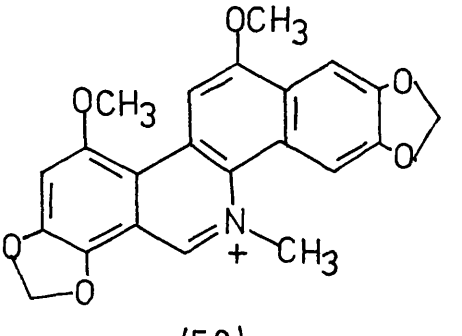
DERIVATIVES WITH MORE THAN FOUR OXYGEN FUNCTIONS

Initially these natural products were thought to be 11-methoxy compounds and it was possible to propose a very simple biosynthesis which involved methylation by a physiological methylating agent, such as methionine. This intermediate would then proceed to the fully unsaturated benzo(c)phenanthridine by the same route as that proposed for sanguinarine (qs).

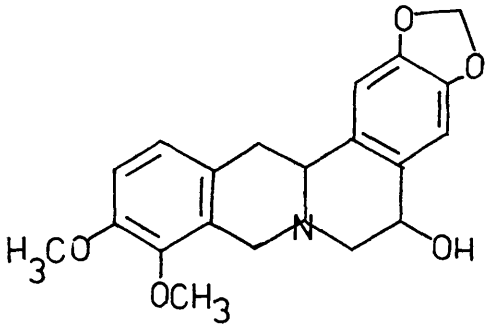
Recent work by Ishii[4] has, however, cast some doubt on the structure of this sub group of compounds and it would appear that the hydroxyl group at C-11 is not methylated but lost in the same way as for sanguinarine etc. The third oxygen function in Ring A may be inserted relatively late in the biosynthesis or it may be present in the biological "Starting materials". From a re-rationalisation of the existing biogenetic studies on macarpine (19) the former is more likely. Although the structure was originally thought to be (19) the work still stands for structure (58), the most likely one in the light of current research.



(19)

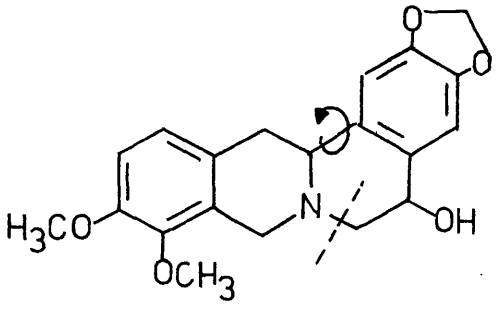


(58)

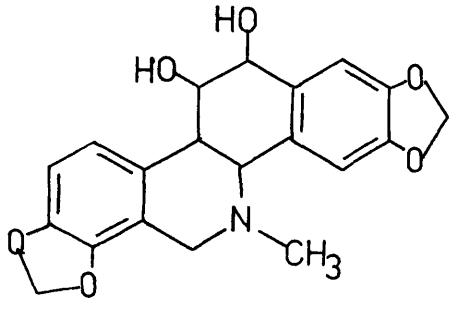


(59)

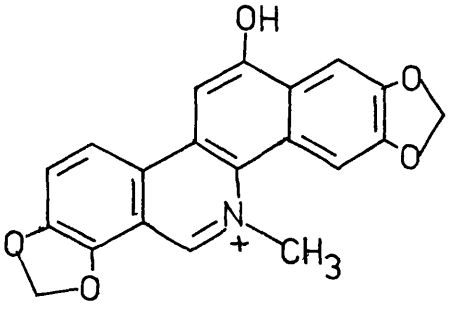
Macarpine is believed to be derived from berberastine (59)



Subjecting this tetrahydroprotoberberine to the biosynthesis described for chelidonine would lead to the dihydroxybenzo(c)phenanthridine (60)

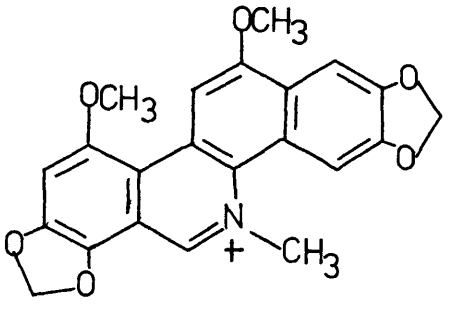


(60)



(61)

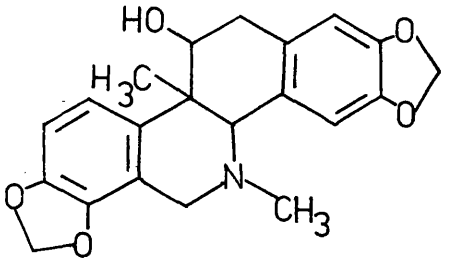
In the original theory, methylation followed by oxidation/ aromatisation led to macarpine; it would seem more likely that oxidation takes place first, producing the intermediate (61)



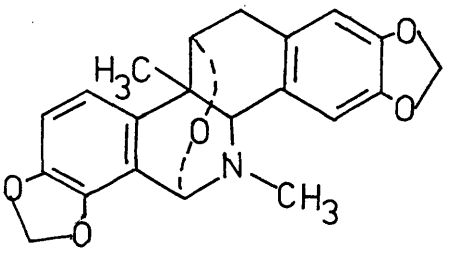
(58)

Hydroxylationof ring A could be followed by O-methylation to produce structure (58). The involvement of berberastine in the biosynthesis of macarpine has been shown[34,35] for Hydrastis canadensis in a series of labelling experiments: this would imply that ring A hydroxylation occurs after tetrahydroberberine formation.

13-methyl derivatives

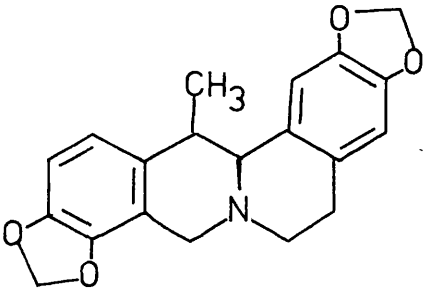


(25)



(30)

Again, these derivatives are assumed[9] to arise from the mechanism postulated above. Circumstantial evidence is provided by the occurrence of the compound (62)



(62)

with corynoline (25) and corynoxoline (30) in the plant Corydalis incisa.[10,36]

Two in-vitro routes to 13-methyldihydroprotoberberines are known, one involving methylation of the 1-benzylisoquinoline precursor,[37] the other a methylation of the dihydroprotoberberine,[38]

the latter being preferred[28] as a model for biosynthesis. Tracer experiments are inconclusive.[39]

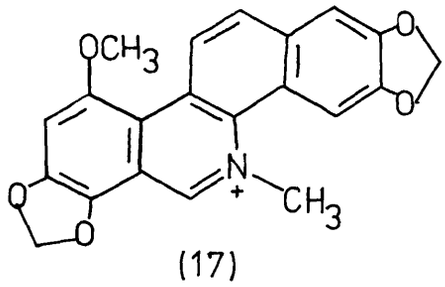
STRUCTURE AND STEREOCHEMISTRY

Structure of fully unsaturated compounds

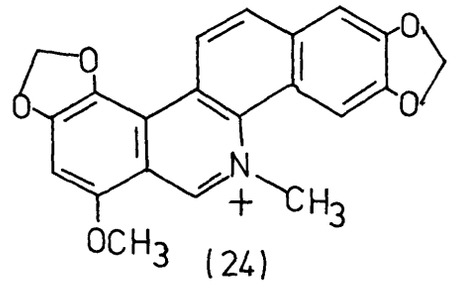
The structures of sanguinarine (6), chelerythrine (7), chelidonine (20) and homochelidonine (21) were elucidated by classical degradative techniques in the 1930s by Bruchhausen and Berch[40] and by Späth and Kufner[41], and have been the subject of several reviews.[42,43,44] All have been confirmed by synthesis.[45,46]

The structures of avicine (8) and nitidine (9) have also been elucidated by degradative techniques, by Arthur, Hui and Ng.[2] Confirmatory syntheses have been carried out.[47,48]

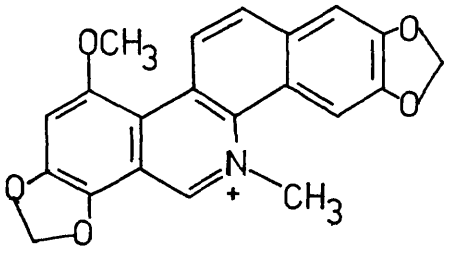
More modern techniques have been used in elucidation[5] of the structure of bocconine (24) by the comparison of the proton n.m.r. spectrum of its 5,6-dihydro derivative with that of dihydrosanguinarine and dihydrochelerythrine. This still left some doubt as to the substitution in Ring A but use of the nuclear overhauser effect led to the choice of (24) rather than (17)



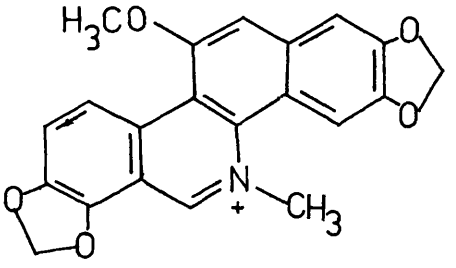
(17)



(24)



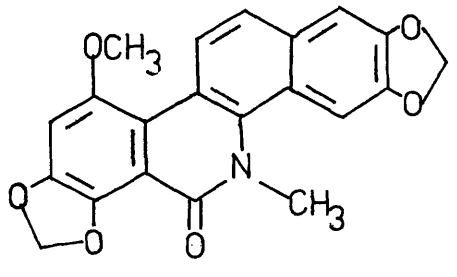
(17)



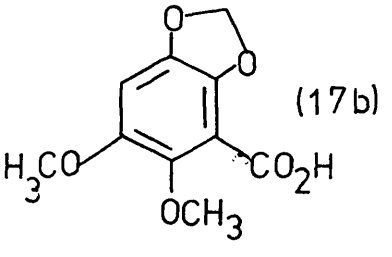
(15)

since irradiation of the methoxy signal led to an increase in the Ring A proton resonance but had no effect on the resonance of the C-11 proton.

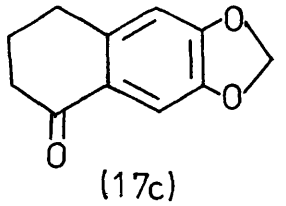
Recent work by Ishii and his group[4] has, however, shown that (17) is the correct structure and that (17) and chelirubine (15) are also identical. This re-assignation was again done using proton magnetic resonance but was also supported by an unambiguous synthesis of oxychelirubine (17a), from the starting materials (17b) and (17c), in four steps



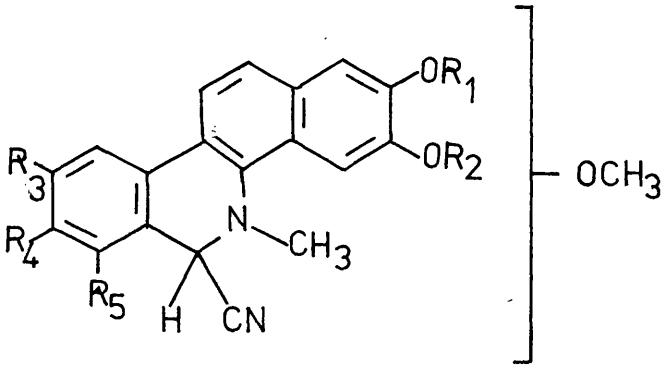
(17a)



(17b)



(17c)



	R_1	R_2	R_3	R_4	R_5
(13)	CH_3	CH_3	H	$O-CH_2-O$	
(14)	CH_3	CH_3	H	OCH_3	OCH_3
(15)	CH_2		H	$O-CH_2-O$	
(16)	CH_2		H	OCH_3	OCH_3

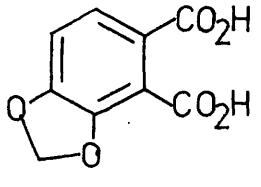
The structure of the four compounds, chelirubine (15), chelilutine (16), sanguirubine (13) and sanguilutine (14) were originally assigned on the basis of their infra red spectra and these were later corroborated by Slavik[49], who examined the proton n.m.r. spectra of the 5,6-dihydro-6-cyanide derivatives. The work by Ishii on chelirubine must cast doubt on the structures of the other three compounds and also on the dimethoxy alkaloid macarpine (19). These compounds remain to be investigated.

Structure of partially unsaturated compounds

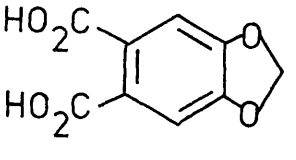
The structural and stereochemical implications of the partially unsaturated alkaloids are well illustrated by chelidonine (20). The classical degradative work was carried out by Bruchhausen and Bersch.[40]

The molecular formula was found to be $C_{20}H_{19}NO_5H_2O$ and was found to comprise two methylenedioxy groups, one N-methyl group and one hydroxyl group; the carbon skeleton was identified when benzo(c)phenanthridine was obtained after distillation of the alkaloid with zinc dust. Oxidation of chelidonine with potassium permanganate gave a mixture of 3,4- and 4,5-methylene-dioxyphthalic acids (63) and (64)

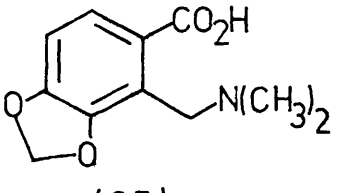
Hofmann degradation gave a methine which was degraded by potassium permanganate to 4,5-methylenedioxyphthalic acid and the amino acid (65)



(63)

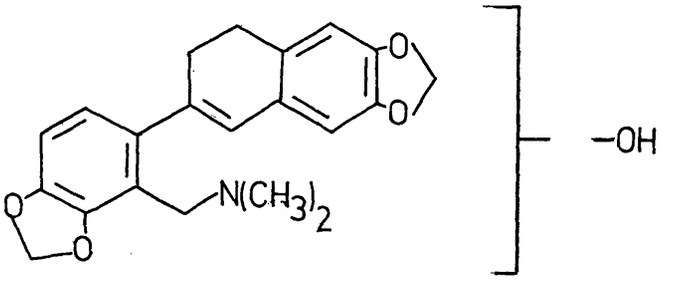


(64)



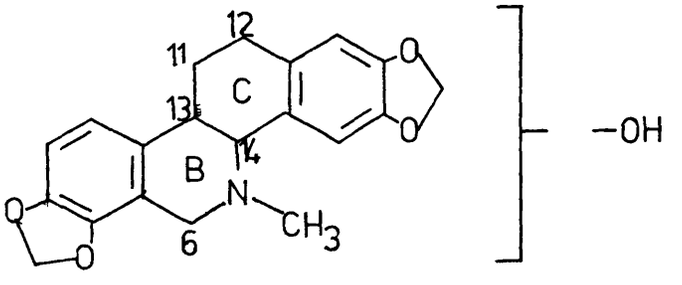
(65)

From this evidence it was possible to infer the part structure (66) for the methine



(66)

and the part structure (67) for chelidonine.



(67)

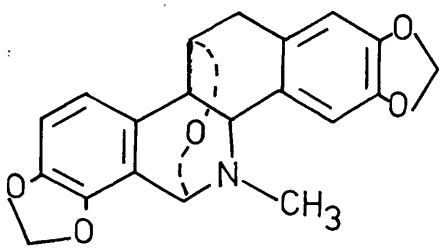
This leaves only the position of the hydroxyl group and the orientation of the B–C ring junction to be decided. Since the hydroxyl group possessed no phenol properties it must be on ring B or C. Positions 6 and 14 were discounted since no carbinolamine properties were apparent and position 13 because the hydroxyl group was retained in the methine.

Thus the hydroxyl could be situated at either C11 or C12, both of which would give similar reactions.

Oxidation of chelidonine by mercuric acetate gave a mixture of products including didehydrochelidonine, recently identified by Benn and Mitchell as (68),[Can. J Chem., 1969, 47, 3701.]

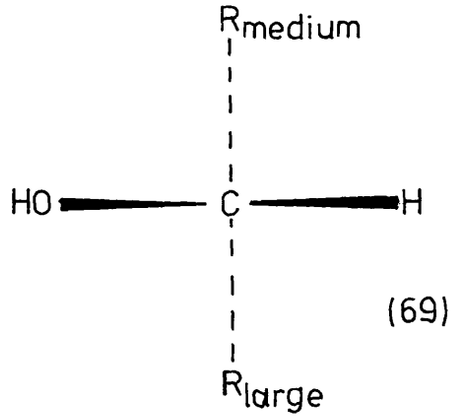
Overall the chemical evidence supports the structure (20) with the hydroxyl group at C-11. Proper confirmation of this was obtained by Bersch[51] by considering the IR spectrum which showed a strongly hydrogen bonded OH absorption. Bersch reasoned that this degree of hydrogen bonding was only possible if the OH group was in the C-11 axial position with the B and C rings cis-fused. This has since been confirmed by n.m.r.[52,53,54] studies which also showed that chelidonine adopted the half chair conformation.

The absolute stereochemistry of chelidonine was investigated by Snatzke et al[55] using two methods. The first method was based on that originally developed by Horean, which correlated the absolute configuration of secondary alcohols with the kinetic partial resolutation during esterification with a α-phenylbutyric anhydride. An excess of (±)-α-phenylbutyric anhydride was added to the optically active secondary alcohol. Unreacted anhydride was recovered and hydrolysed and the optical



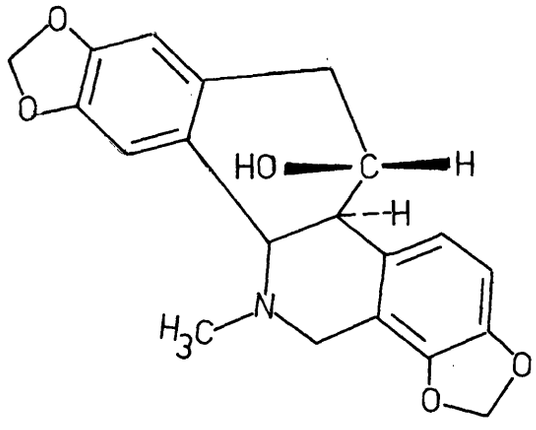
(68)

rotation measured. It has been shown empirically that if the alcohol is written as (69)



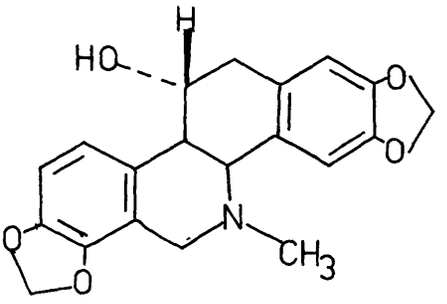
(69)

then the recovered acid has the (S)(+) isomer in excess. Thus, chelidonine was found to be(71)



(71)

This is equivalent to (71a) since the relative configurations are known.

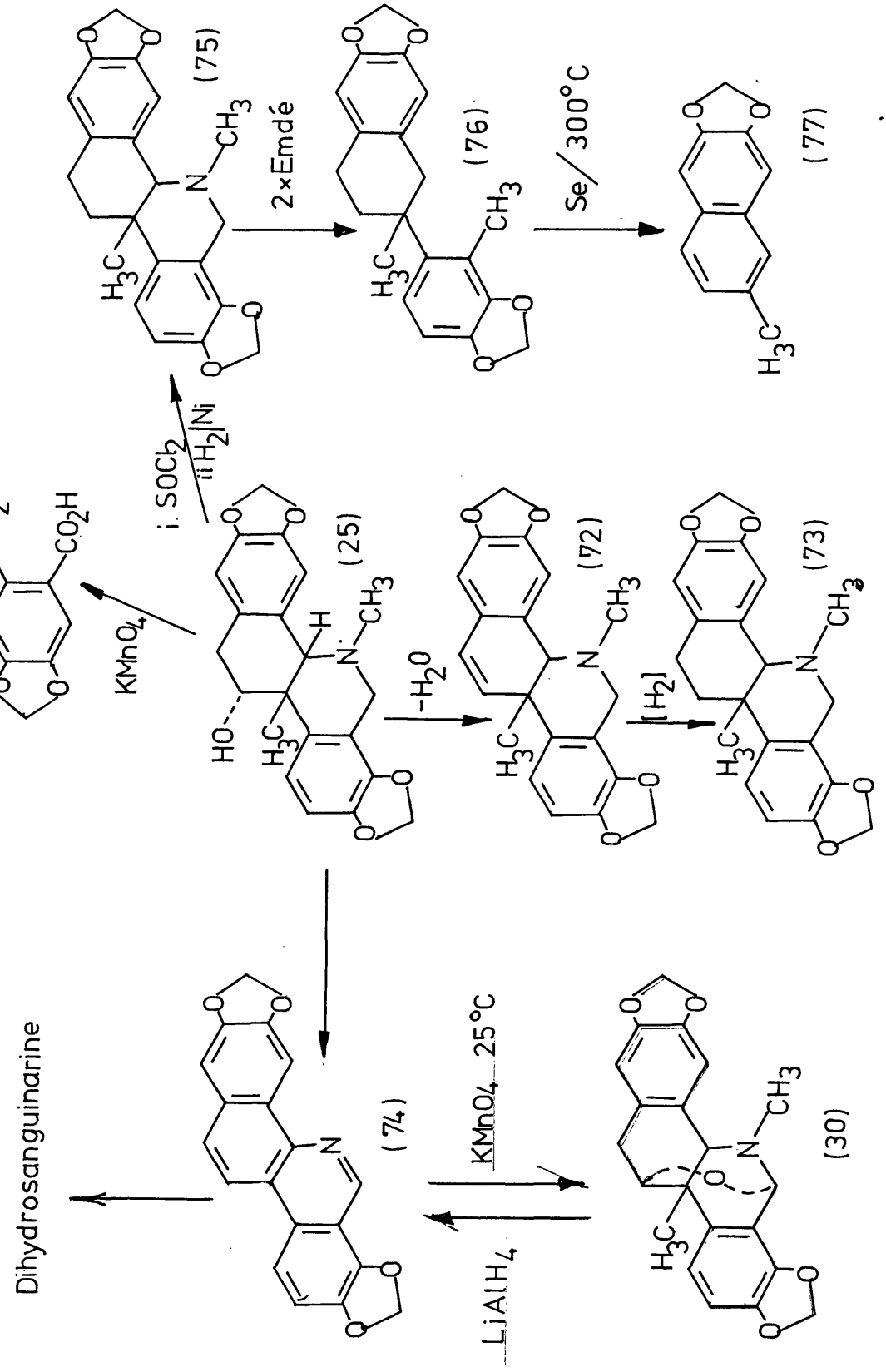


(71a)

This empirical study was supported by an analysis of the c.d. spectrum of chelidonine, in which the same conclusions were reached.

The final proof of the structure was provided by the total synthesis of (±) chelidonine carried out by Oppolzer and Keller[56] (qv). More recently the structures of corynoline (25) and corynoloxine (30) were determined by Takao[57] using degradative and spectroscopic techniques.

The molecular formula of corynoline was found to be $C_{21}H_{21}NO_5$ and the molecule was found to contain one alcoholic hydroxyl group, one N-methyl, one O-methyl group and two methylenedioxy functions. Corynoline was readily dehydrated to form deoxycorynoline (72) which was hydrogenated to dihydrodeoxycorynoline (73). Heating corynoline <u>in vacuo</u> at 300°C gave the benzo(c)phenanthridine (74) with C-demethylation, which was transformed into dihydrosanguinarine, thus fixing the


Dihydrosanguinarine
(74)
KMnO4 25°C
LiAlH4
(30)
(25)
KMnO4
i. SOCl2
ii H2/Ni
(75)
2×Emdé
(76)
Se/300°C
(77)
-H2O
(72)
[H2]
(73)

position of the methylenedioxy groups.

Removal of the hydroxyl group by chlorination and hydrogenolysis led to (75) which, after two Emdé degradations gave the dimethyl compound (76). Heating (76) with selenium at 300°C produced 2,3-methylenedioxy-6-methylnaphthalene (77): this fixes the C-methyl group at C13. The hydroxyl group was placed at C-11 by analogy with chelidonine. When corynoline was oxidised by aqueous potassium permanganate, corynoloxine (30) was formed. This (a) shows the relationship between the two natural products and with sanguinarine; (b) indicates that the B and C rings are cis-fused as in chelidonine. The fusion was also indicated by the intramolecular hydrogen bonding shown in the IR spectrum.

The stereochemistry has been considered by Naruto, Arakawa and Kaneko[54] and their proposals confirmed by an X-ray analysis of the p-bromobenzoate carried out by Kametani.[58]

SYNTHESIS

Several approaches to the synthesis of benzo(c)phenanthridine alkaloids have been used since the first attempts early in this century. These are summarised as follows:-

i) Chrysenequinone

ii) Bischler-Napieralski

iii) Homophthalimide

iv) 1,2-dihydroisoquinoline

v) Photochemical cyclisation

vi) Isocoumarin

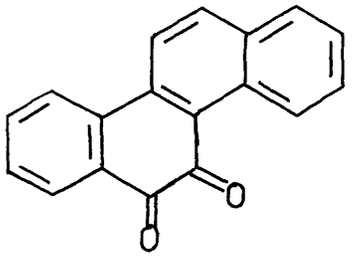
vii) Pschorr ring-closure

viii) Benzyne reaction

ix) Total synthesis of chelidonine (intramolecular cycloaddition)

i) Chrysenequinone

The route from Chrysenequinone (78) is of historic importance only, the original work being done by Graebe and Honigsberger[59] at the turn of the century.



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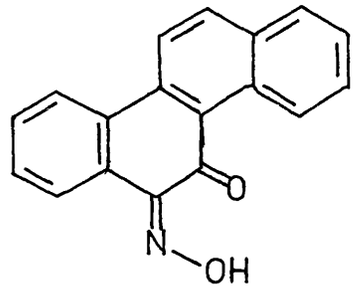
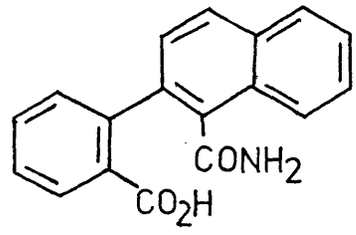


(79)

The amide (80) was formed by rearrangement of the oxime (79) of Chrysenequinone. On treatment with sodiumhypochlorite the amide produced the isocarbostyril (82) which in turn gave the parent ring system upon zinc dust distillation.

(79) ⟶



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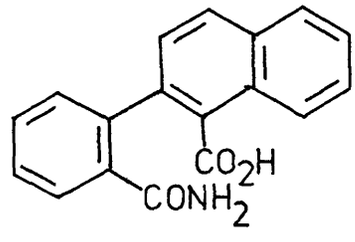

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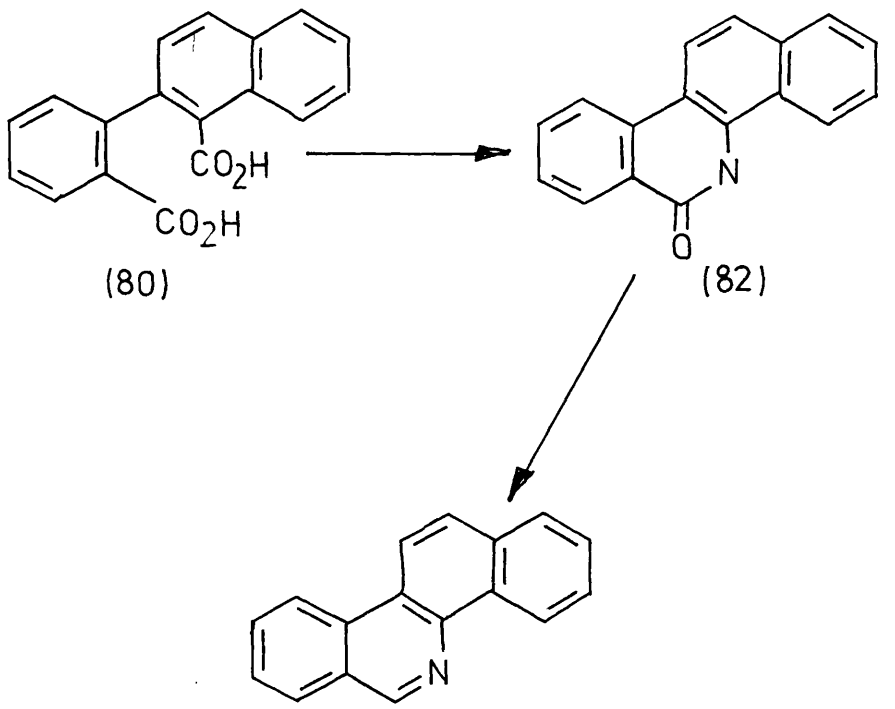


Benzo(c)phenanthridine

The route was further investigated by Badger and Seidler[60] who prepared the isocarbostyril from the amide using hydrazoic acid.

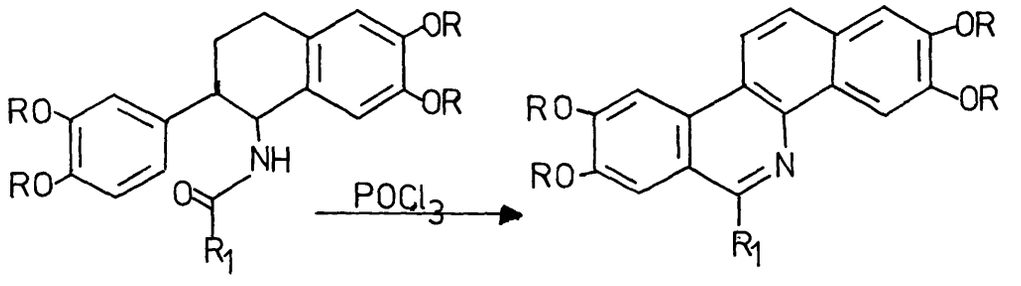
As a route to the naturally occurring benzo(c)phenanthridine alkaloids this has several drawbacks, the main one being the considerable difficulty in producing chrysenequinone derivatives of suitable oxygen functionalisation.

ii) Bischler-Napieralski

The Bischler-Napieralski approach is the next chronologically and was the approach utilised by Robinson and his group,[61] and later, by Govindachari et al,[62,63] and Arthur and Ng.[2]

Robinson's route to the 2,3,8,9-tetramethoxybenzo(c)-phenanthridine is shown in Scheme 2.

This type of ring closure was used by Govindachari et al[62,63] to synthesise several 6-methylbenzo(c)phenanthridines and by Govindachari and his co-workers,[47,64] and by Arthur and Ng,[2] independently, to synthesise dihydronitidine (37).



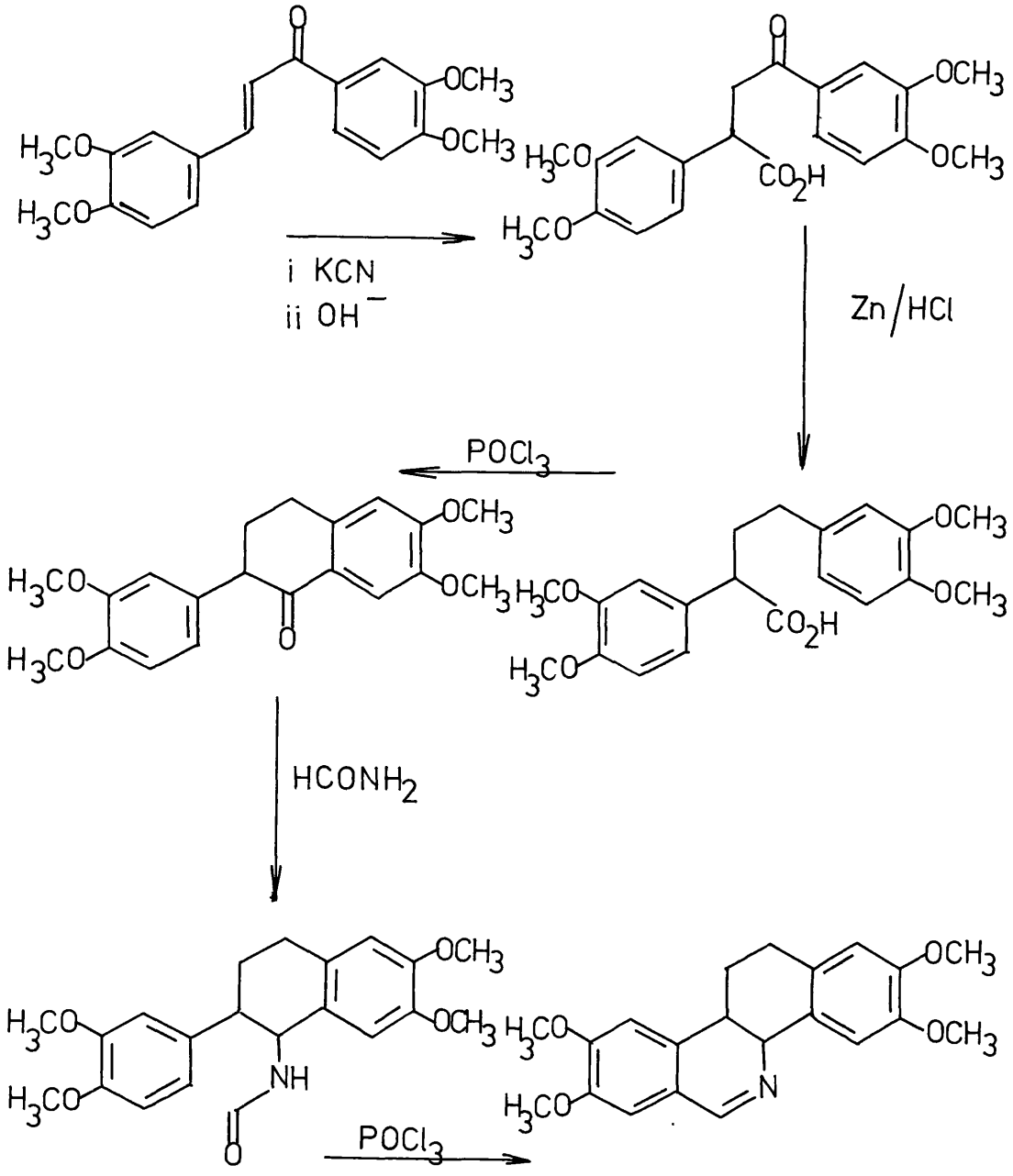
R = H, CH_3

R = H, alkyl

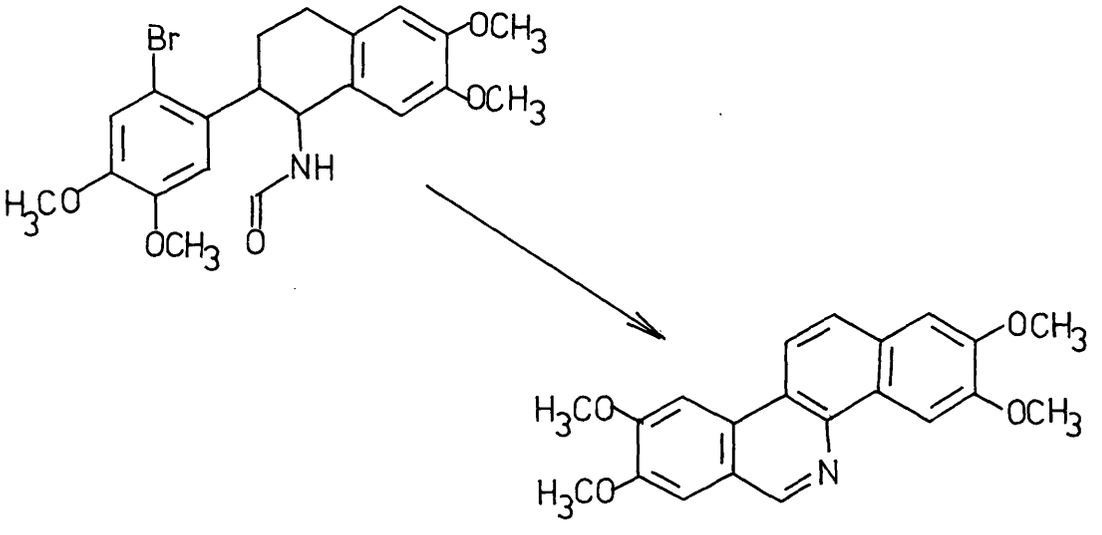
It can be seen that all the derivatives described above have the same, symmetrical 2,3,8,9-oxygenation pattern and it has been found that in practice the Bischler-Napieralski approach does not lend itself to the synthesis of 2,3,7,8-oxygenated products. Indeed, the attempt by Robinson et al[65] to produce the unsymmetrical compound by the introduction of a

SCHEME 2

Synthesis of 2,3,8,9 tetramethoxybenzo(c)phenanthridine

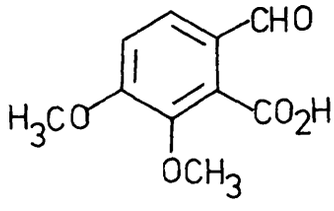


nuclear bromine blocking group produced the symmetrical product with elimination of bromine.

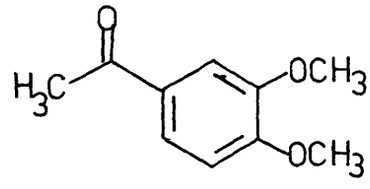


iii) Homophthalimide route

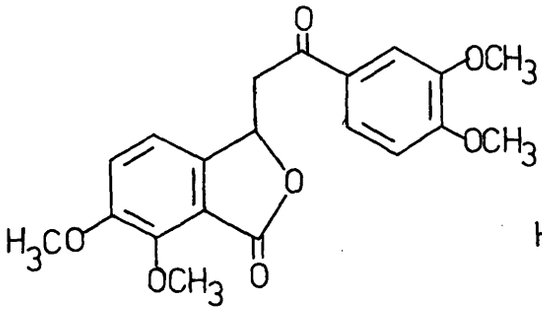
This route was developed by Robinson[65,66] after the unsuccessful attempts mentioned above to produce the unsymmetrically oxygenated benzo(c)phenanthridines by the Bischler-Napieralski route. In this synthesis the oxygenation pattern in the A ring is fixed by condensation of opianic acid (83) and the ketone (84) to give the phthalide (85), followed by a multistep synthesis to give the homophthalimide (86).



(83)



(84)



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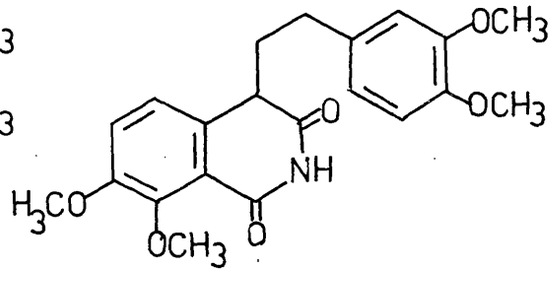
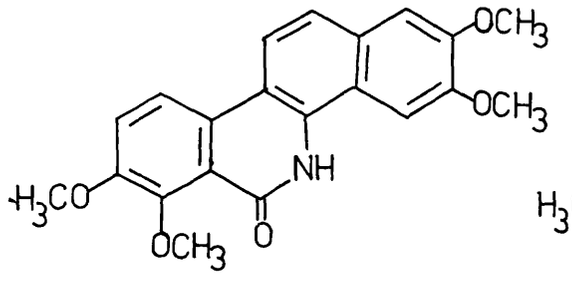


(86)

After polyphosphoric acid cyclisation of the homophthalimide to give the isocarbostyril (87), the fully aromatic compound (88) was obtained.



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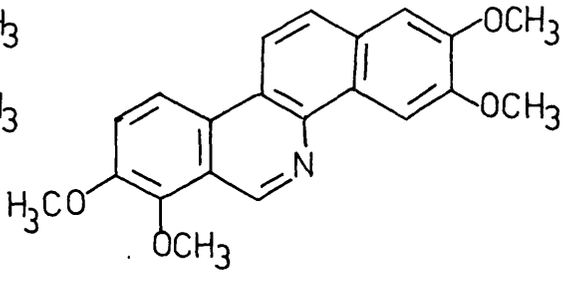
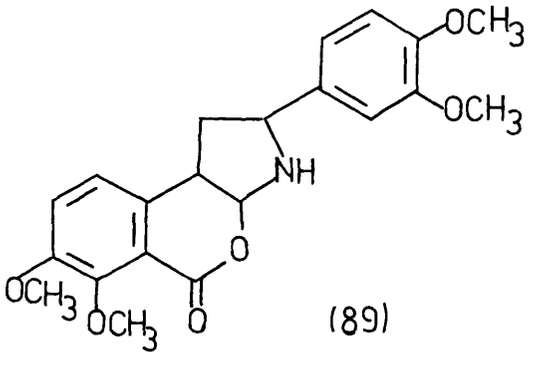


(88)

Two factors prevent the wider use of this synthetic route:

i) addition of potassium cyanide to the phthalide (85) forming the intermediate (89) prior to hydrolysis to the diacid (90) is difficult and extremely sensitive to reaction conditions.[66,67]



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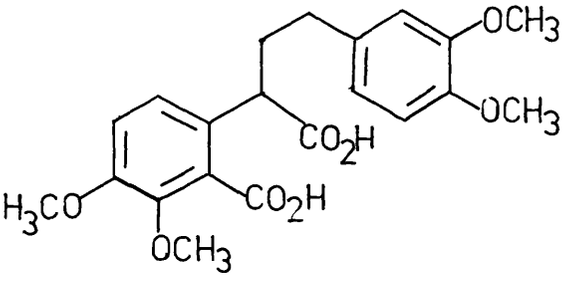
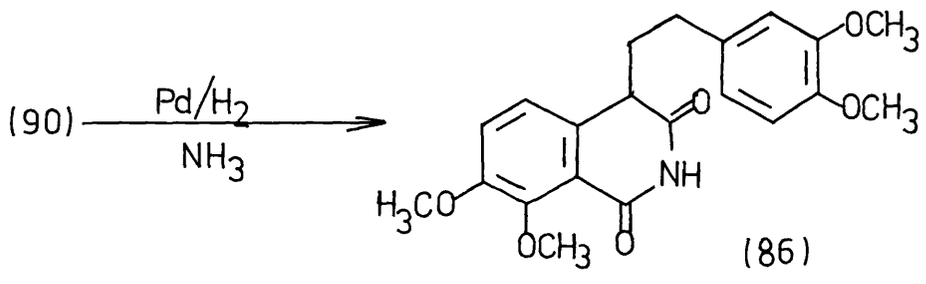


(90)

ii) The catalytic reduction conditions used to form the homophthalimide are sufficient[45,67,68] to cleave some ethers, especially

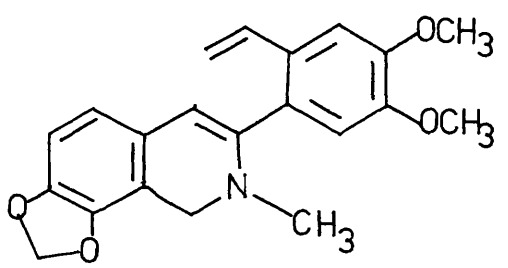
the cyclic methylenedioxy group. This severely limits the use of the route since it precludes the synthesis of a large proportion of the naturally occurring alkaloids.



(86)

iv) 1,2-dihydroisoquinoline

Since it has been shown above that it is likely that chelidonine (19) is formed *in-vivo* via a 1,2-dihydroisoquinoline derivative it has occurred to several workers to attempt an *in-vitro* synthesis by this approach. Robinson[65] attempted to convert anhydrocryptopine (91) to the benzo(c)phenanthridine by treatment with acid but this reaction was shown by Dyke and Brown[69] to give the 11-methyl-indeno(1,2-c)-isoquinoline (92).



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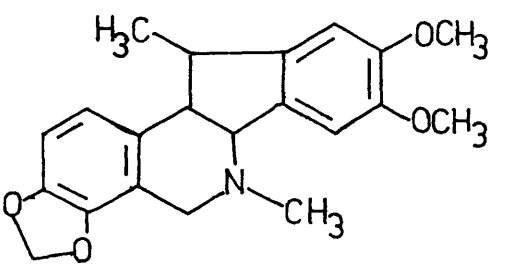
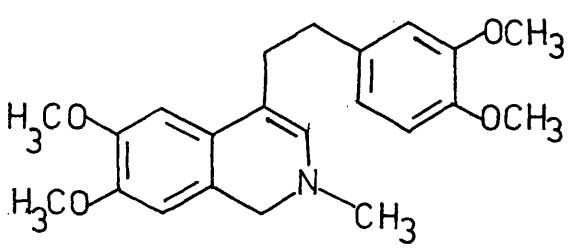
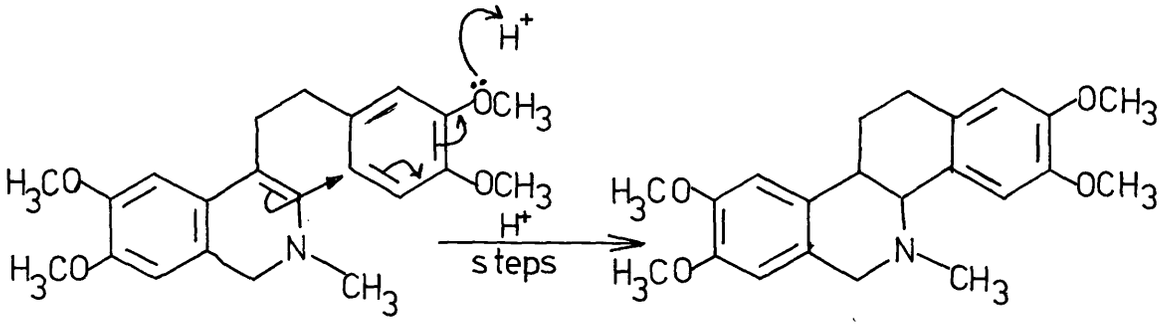


(92)

In another piece of work, Dyke and his co-workers[70,71] produced compounds of the type (93) and attempted the acid-induced ring--closure.



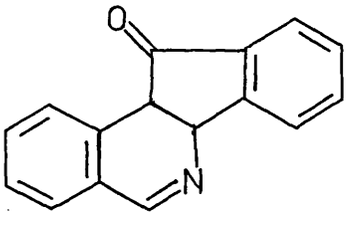
(93)



(93)

Although this reaction had been expected[72] to produce the benzo(c)phenanthridines, Dyke and his team observed only disproportionation.

Dyke and his co-workers[73,74], using the 11-keto- derivatives (94) of indeno-(1,2-c)isoquinolines, formed from the reaction of aromatic aldehydes with 1,2-dihydroisoquinolines followed by oxidation, attempted the diazomethane ring-expansion to the benzo(c)phenanthridines but met with no success.



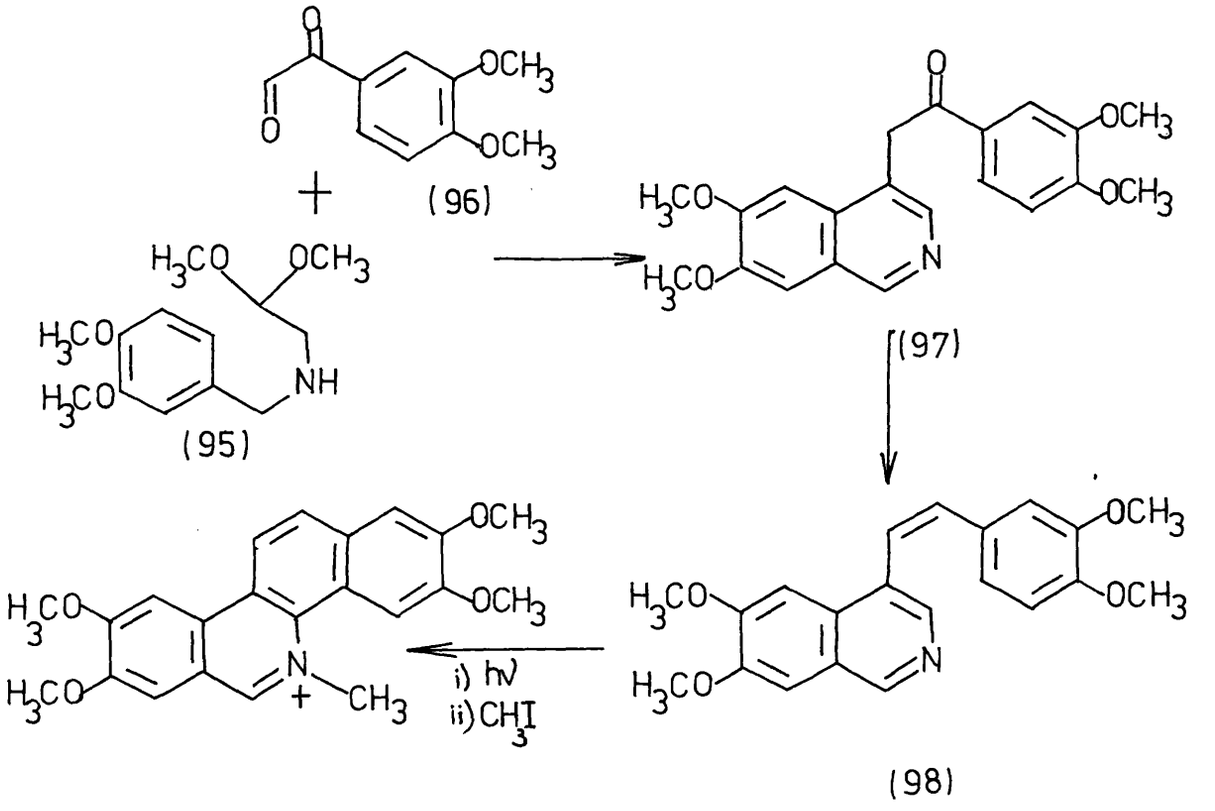
(94)

Work carried out by this author on this reaction is described later in this thesis.

v) Photochemical methods

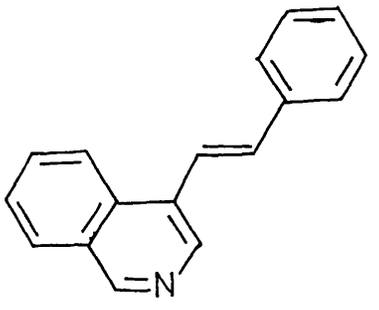
Considerable work has been done on the formation of the benzo(c)phenanthridine skeleton by photochemical methods, particularly by the Japanese group of Onda[75], by Timmons and Loader[76] and by Dyke and his group[77]. Some success has been achieved although the method has yet to be used to advantage in the synthesis of the naturally occurring 7,8-dioxygenated compounds.

The method of Dyke and Sainsbury[77] utilised a 1,2-dihydroisoquinoline. Condensation of the dimethoxybenzylaminoacetal (95) with the dimethoxyglyoxal (96) gave the isoquinoline derivative (97)



which, after sodium borohydride reduction and acid catalysed dehydration gave the styrene (98); irradiation in ethanol solution gave 2,3,8,9-tetramethoxybenzo(c)phenanthridine in 30% yield.

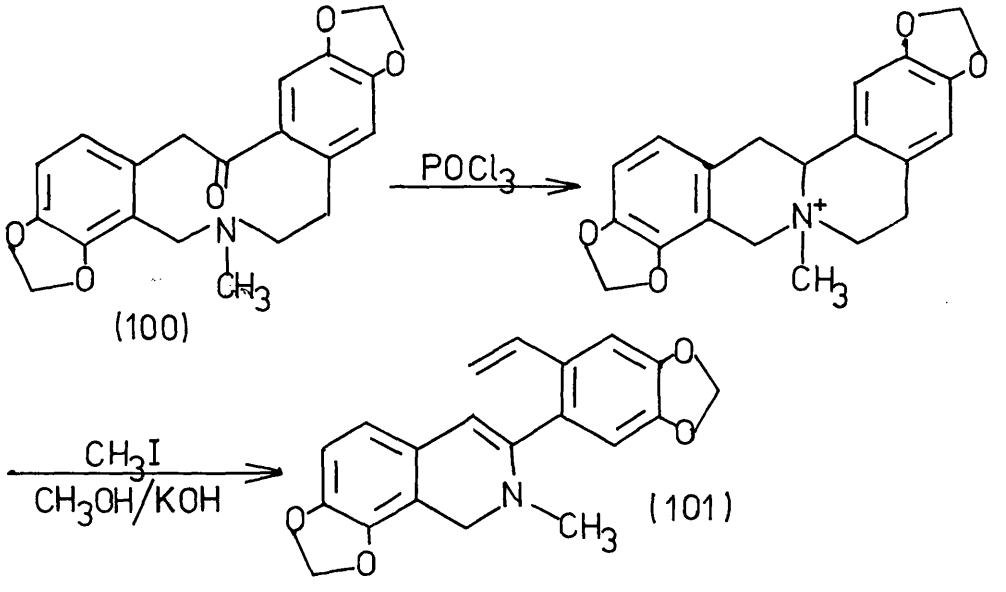
Dyke and Sainsbury used the same method to produce the much less favoured 2,3,7,8-tetramethoxybenzo(c)phenanthridine in extremely low yield. Using a similar approach, Loader and Timmons[76] produced the basic skeleton from the styrene (99).



(99)

The route developed by Onda and his group[75], while not a synthesis ab-initio, illustrates that although the unsymmetrical compounds are in general difficult to prepare photochemically, they can nonetheless be prepared from suitable precursors.

Onda started from the natural product protopine (100) and, by Hofmann degradation, produced the vinyl compound anhydro-protopine (101).

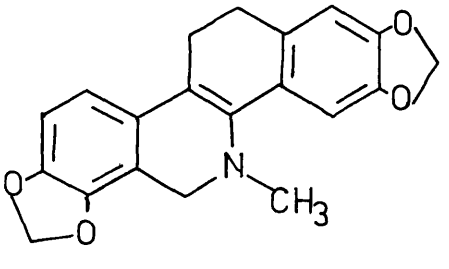


(100)

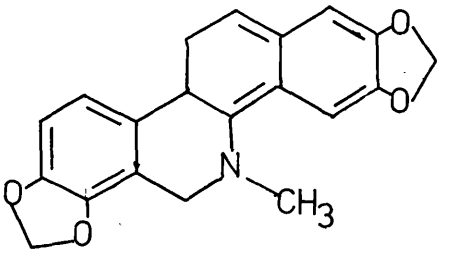
(101)

Irradiation produced an unstable compound, supposed to be a benzo(c)phenanthridine with an unknown degree of saturation in the B and C rings, which was not isolated. On dehydrogenation 5,6-dihydrosanguinarine (25) was isolated and this in turn was oxidised to the fully aromatic compound, sanguinarine.

Onda put forward three structures for the intermediate and offered spectroscopic evidence for the 5,6,11,12-tetrahydro-benzo(c)phenanthridine (102) formed by isomerisation of the initial cycloaddition product (103).

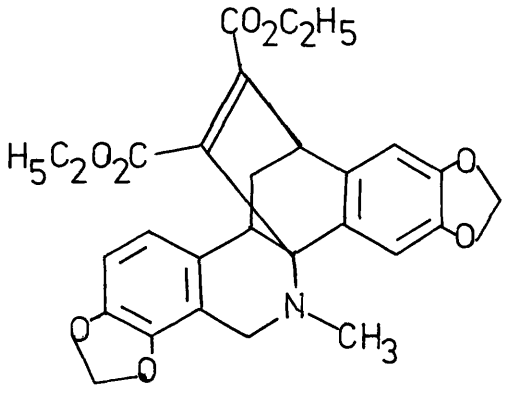


(102)



(103)

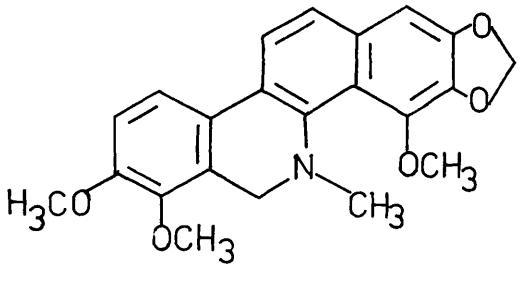
Evidence was provided for structure (103) by the isolation of the Diels-Alder adduct (104)



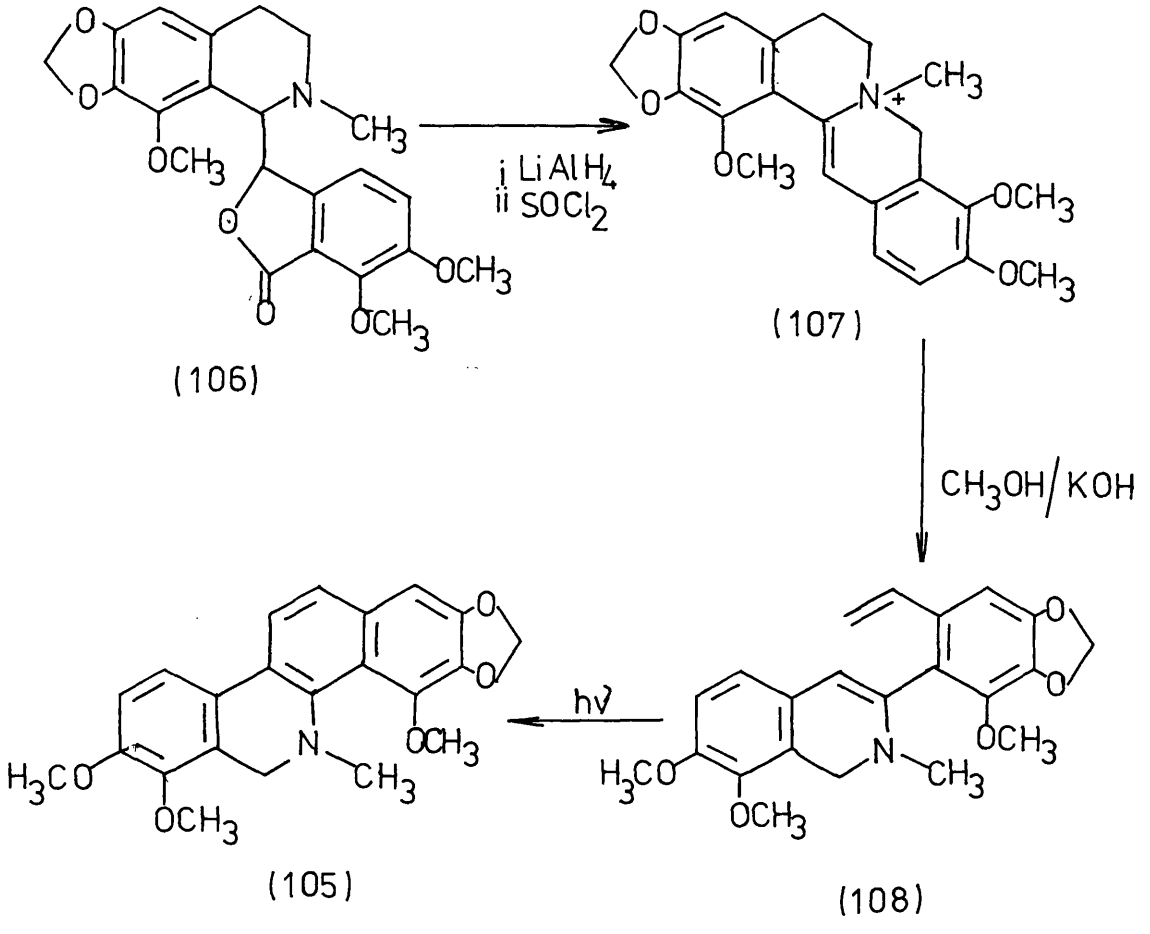
(104)

formed by addition of diethylacetylenedicarboxylate to the reaction mixture.

In another example of this type of synthesis Onda and Kawakami[78] synthesised the dihydrochelerythrine derivative (105) from 1-α-narcotine (106). Reductive cleavage followed by re-cyclisation gave the dihydroprotoberberinium salt (107) which, on treatment with methanolic potassium hydroxide, produced the methine (108). Photocyclisation followed by dehydrogenation gave (105).

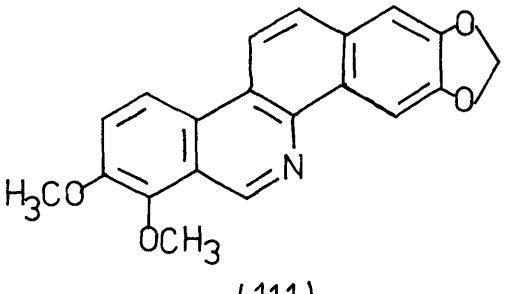


(105)

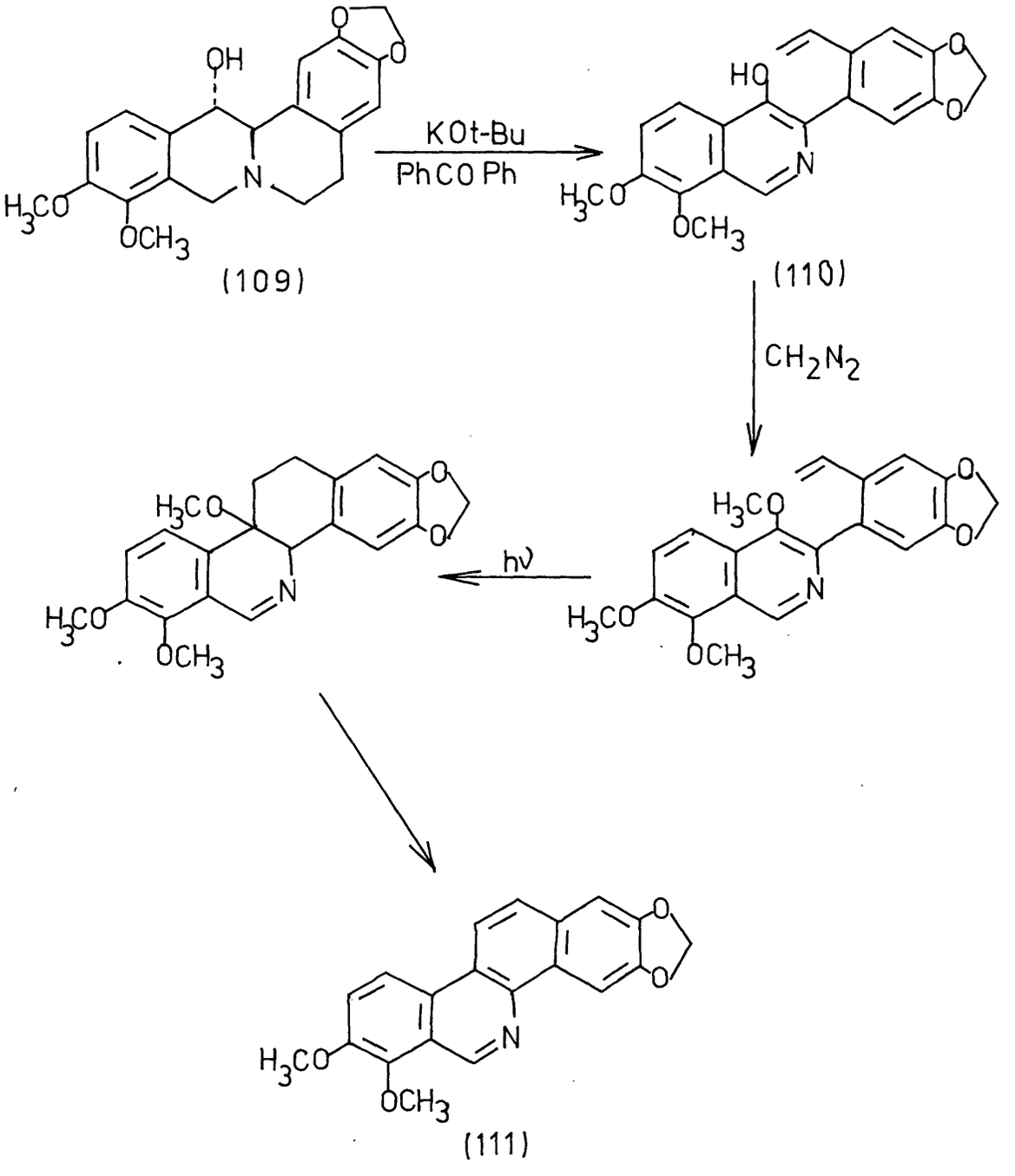


(106) (107) (105) (108)

A simple synthesis of N-norchelerythrine (111) has been developed using this approach by Manske et al[79] starting from (±)-ophiocarpine (109), the initial

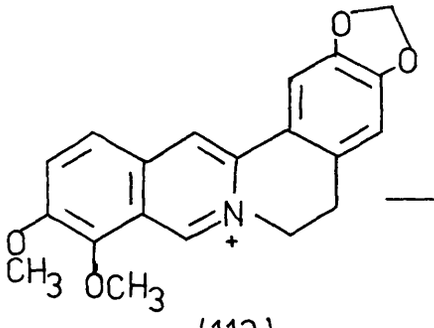


(111)

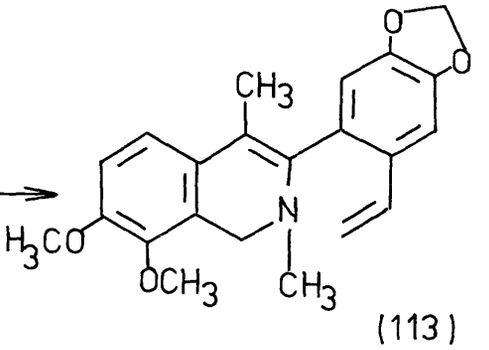


ring cleavage to the methine (110) being brought about using modified Oppenauer conditions. Essentially the same method has been used by Onda to produce a mixture of the two 13-methyl

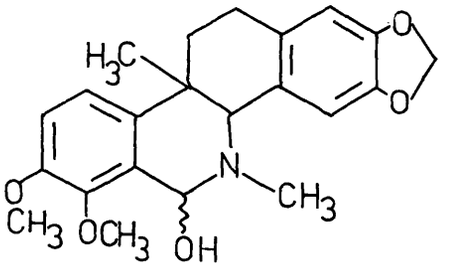
compounds (114) and (115) from berberine (112) via the 4-methyl-1,2-dihydroisoquinoline (113).



(112)

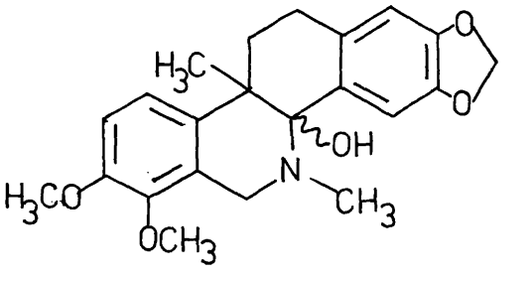


(113)



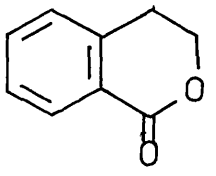
(115)

+



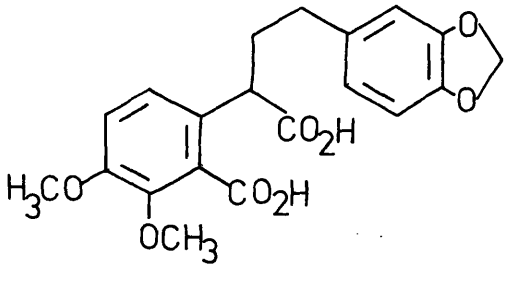
(114)

vi) Isocoumarin

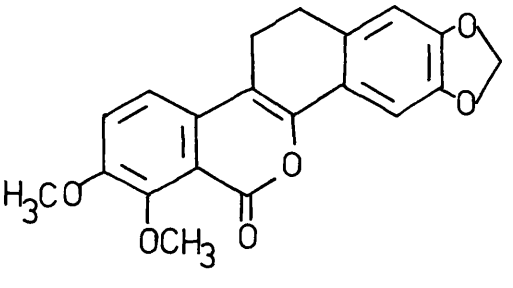


isocoumarin

The potential of a synthetic approach involving the replacement of the oxygen atom of suitably substituted isocoumarin derivatives by nitrogen has long been recognised. Initial work was carried out by Bailey and Worthing[45] who converted the diacid intermediate (116) formed in the Robinson homophthalimide route into the isocoumarin (117).

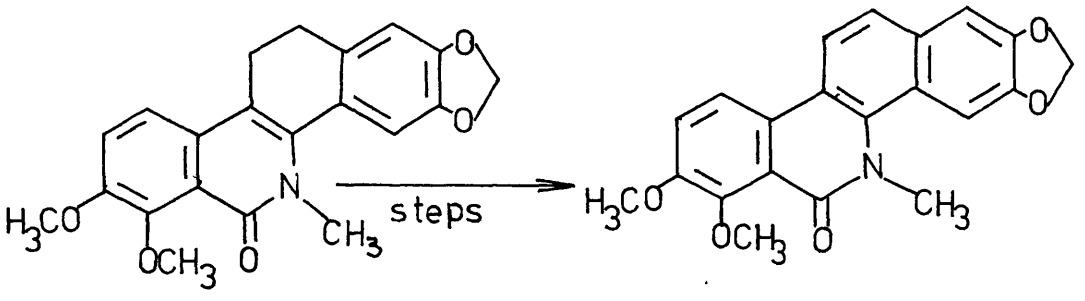


(116)



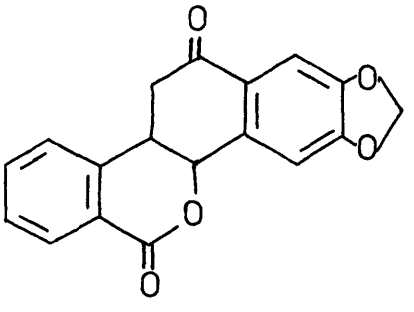
(117)

The isocoumarin thus formed was heated with a mixture of ethylene glycol and ammonia and gave oxychelerythine (118).



(118)

Considerable work has been carried out in this laboratory directed towards the utilisation of isocoumarin derivatives in the synthesis of naturally occurring benzo(c)phenanthridine alkaloids. Using the model compound (119)

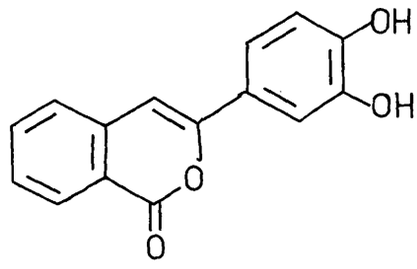


(119)

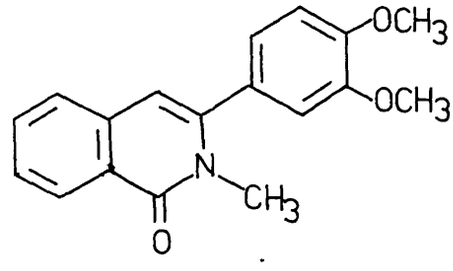
Dyke et al[81] tried unsuccessfully to produce the partially reduced benzo(c)phenanthridine skeleton using both ammonia under pressure and ethanolic ammonia; the former conditions giving only 2-phenylnaphthalene, the latter 2-(β-naphthyl)benzoic acid.

Further work by Wiggins[74] was carried out on the 3-aryl

isoquinolines produced by nitrogen substitution of the 3-aryl isocoumarins formed by the reaction of homophthalic acid, catechol and stannic chloride.



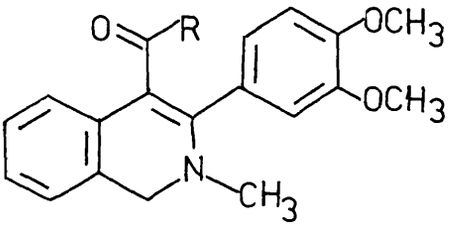
Refluxing with ethanolic methylamine followed by reaction with alkaline dimethylsulphate led to 3-(3,4-dimethoxyphenyl)-2-methylisocarbostyril (120).



(120)

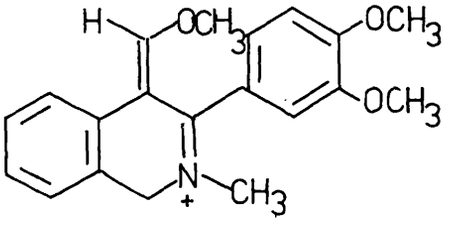
After further exploratory work it was found that the 4-formyl derivative could be formed by a Vilsmeier type of reaction after

reduction to the 1,2-dihydroisoquinoline (121, R=H).

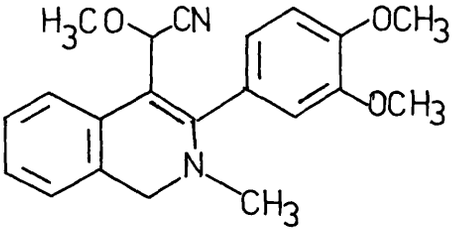


(121)

However, substitution of dimethylacetamide for dimethylformamide in the Vilsmier reaction did not produce the desired 4-acetyl derivative (121, R=Me). Introduction of the second carbon atom (the benzo(c)phenanthridine C-12 atom) was eventually achieved by the introduction of a cyanide group. This was carried out by treating the methoxyiminiumether (122) formed by reaction of the 4-formyl derivative with triethyloxonium tetrafluoroborate with aqueous potassium cyanide viz:



(122)



(123)

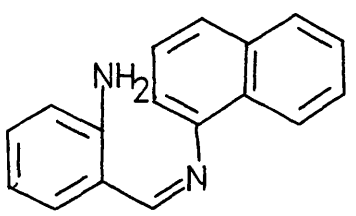
Direct ring-closure of the cyano compound (123) under acid conditions was attempted but cyclisation failed to occur.

Modifications to the cyanide function were also investigated, for example, reduction to the amine as a first step towards Pschorr ring-closure. Several reduction techniques, both complex metal hydride and catalytic, failed to effect the necessary reduction.

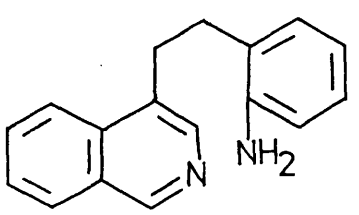
vii) Pschorr reaction

There are two possible applications of the Pschorr reaction to the synthesis of benzo(c)phenanthridine compounds:

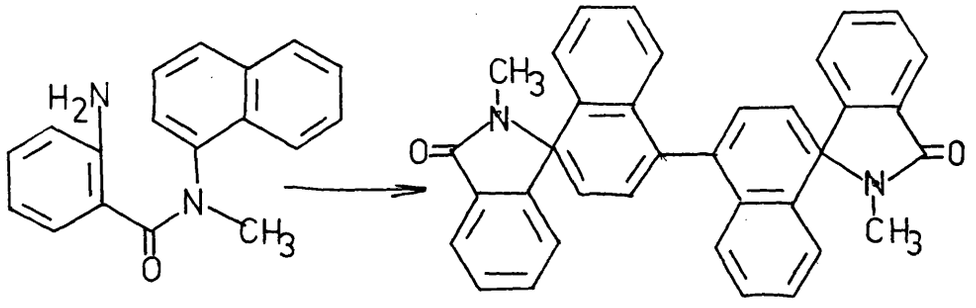
a) formation of ring B



b) formation of ring C

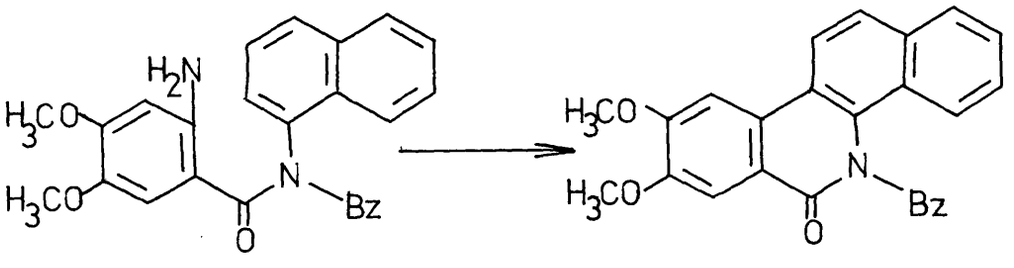


The former application used by Robinson[61], Noller[82], Haworth[68] and Govindachari[83] was unsuccessful due to preferential attack by the radical intermediate of the naphthalene 1-position forming compounds of the type shown below, (124):



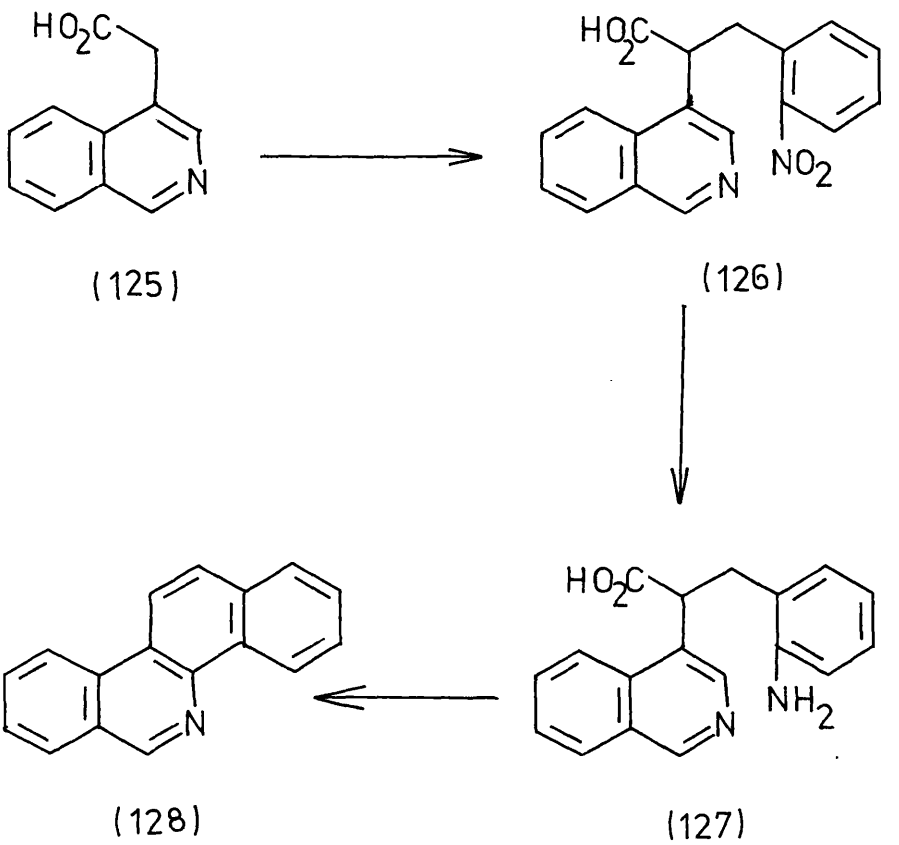
(124)

If the nitrogen holds a group, for example benzyl, which will cause some hindrance to attack at the naphthalene 1-position then attack at the naphthalene 2-position can occur[84], viz:



The more successful approach using the Pschorr reaction is that pioneered by Abramovitch and Tertzakian[85] and subsequently developed into an elegant synthesis of sanguinarine chloride by Dyke and his group[86,87], in which the benzo(c)phenanthridine ring C is completed by Pschorr ring closure.

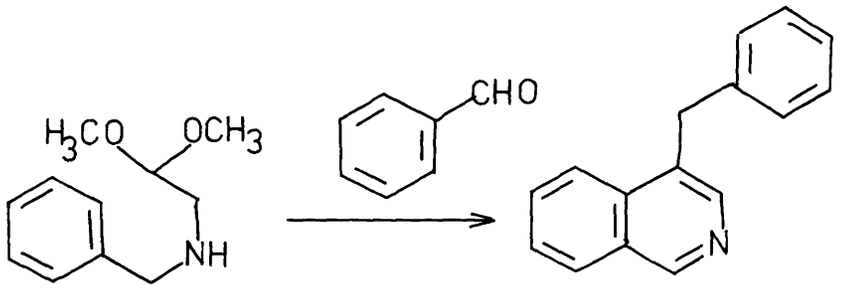
In the original synthesis of benzo(c)phenanthridine by Abramovitch and Tertzakian isoquinoline-4-acetic acid (125) (made by a multistage synthesis) is converted into the amino-acid (127) via the nitroacid (126)



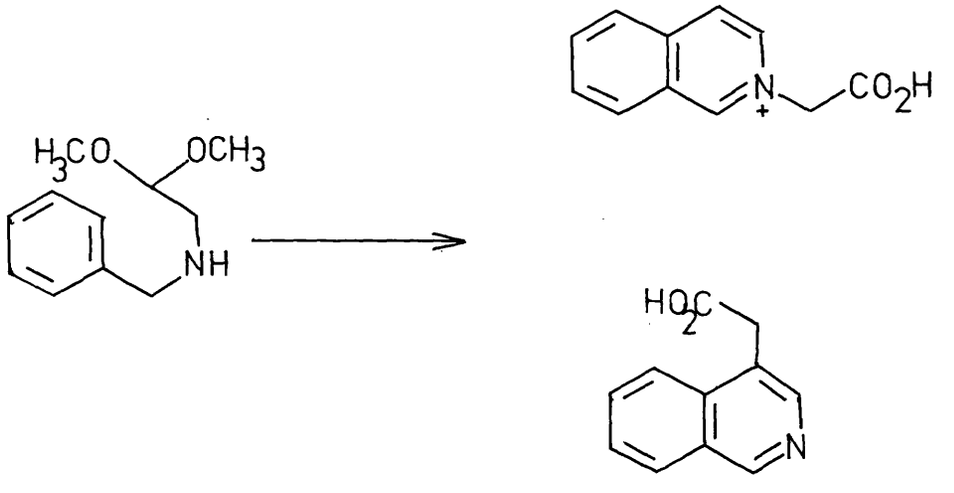
Cyclisation under Pschorr conditions followed by decarboxylation gave benzo(c)phenanthridine (128).

The defect of the synthesis, subsequently overcome by Dyke et al. is the multistage synthesis of isoquinoline-4-acetic

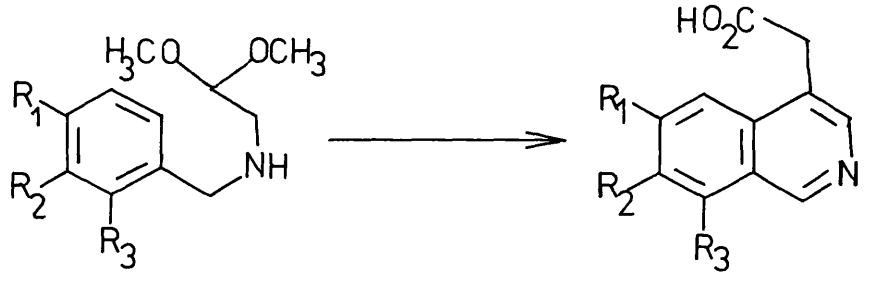
acid: this was only achieved in low yield and not even attempted for oxygenated analogues. Dyke et al.[88] noted the known condensation of N-benzylaminoacetals with aryl aldehydes to give 4-substituted isoquinolines, for example:



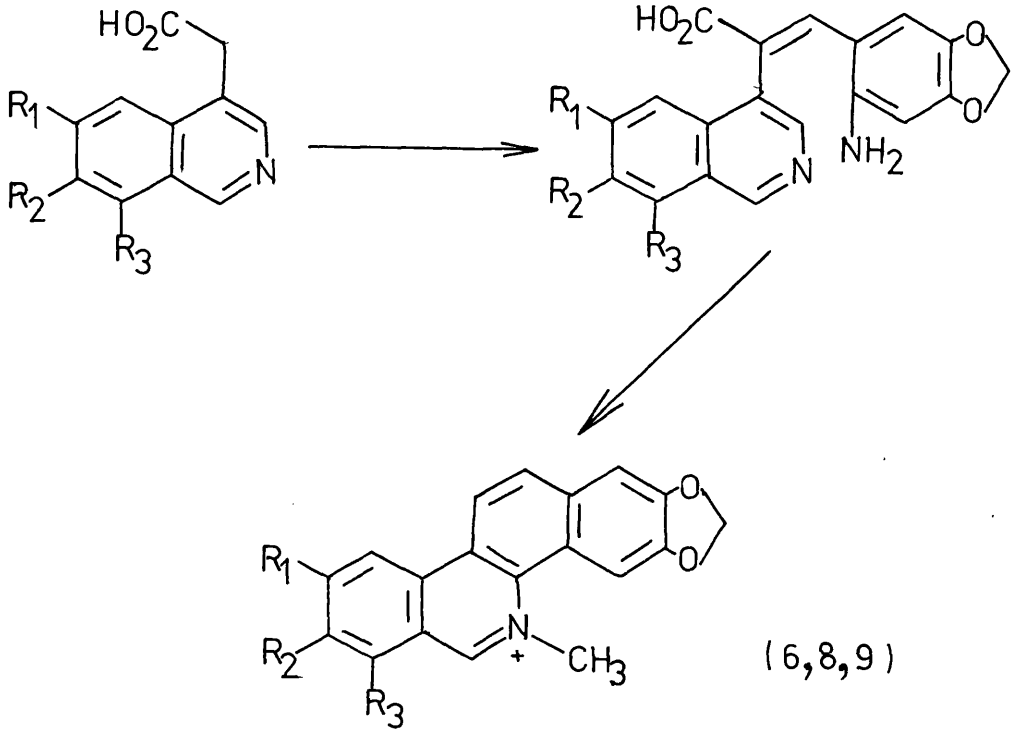
and examined the condensation of aminoacetals with glyoxylic acid: this was found to give an easily separable mixture of isoquinoline 2- and 4-acetic acids:



Reaction with suitable oxygenated aminoacetals allowed the synthesis of avicine (8), nitidine (9) and sanguinarine (6).



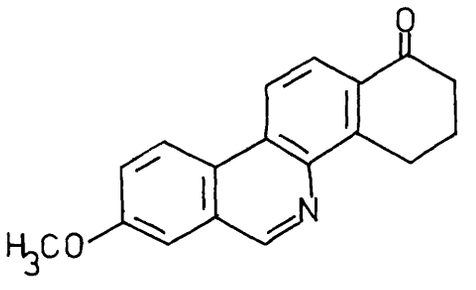
	R_1	R_2	R_3
(8)	$O-CH_2-O$		H
(9)	MeO	MeO	H
(6)	H	$O-CH_2-O$	



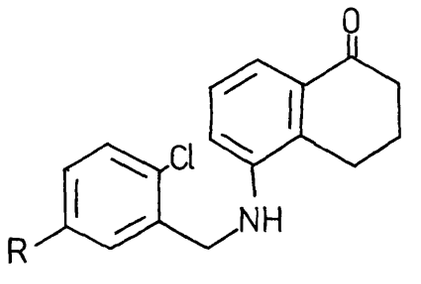
(6,8,9)

viii) Benzyne reaction

Little use has been made of this reaction and the extreme reaction conditions used to generate some aryne intermediates make it unlikely that it will find much utilisation in syntheses aimed at compounds containing the sensitive methylenedioxy function. However, Kessar[89] and his co-workers have produced the 3,4-dihydro-8-methoxybenzo(c)phenanthridin-1(2H)-one (129) by treating (130, R=OCH_3), with potassamide in liquid ammonia in a benzyne reaction.

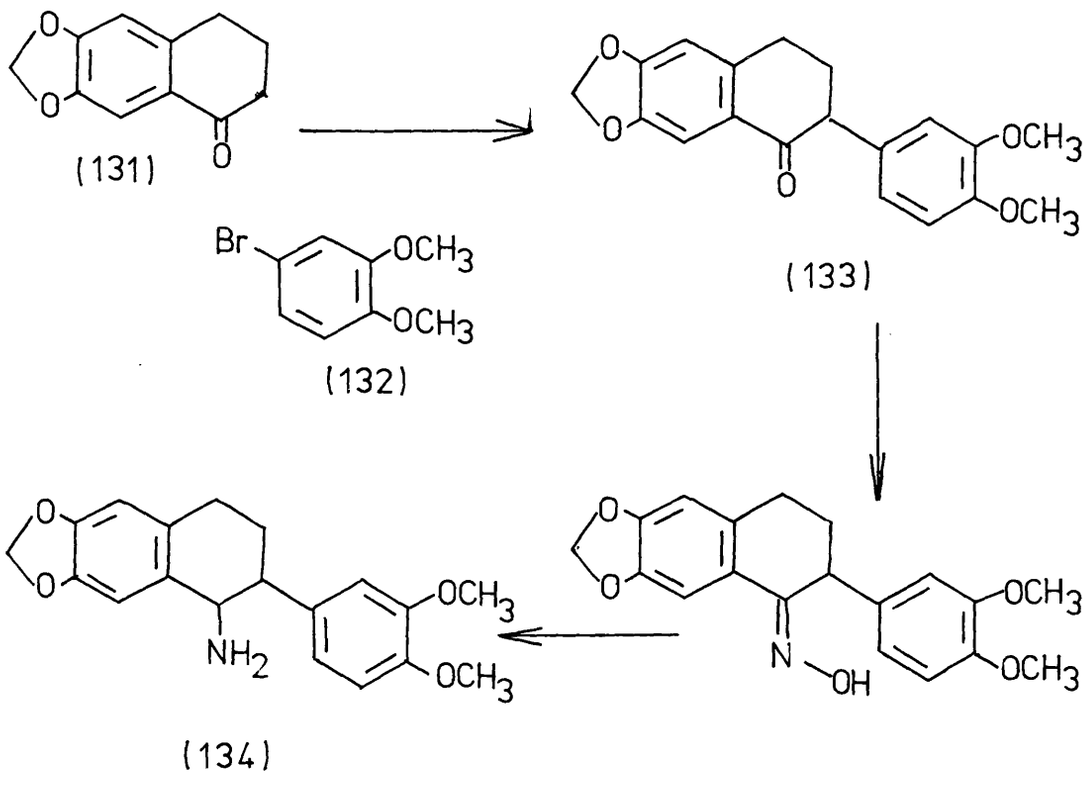


(129)

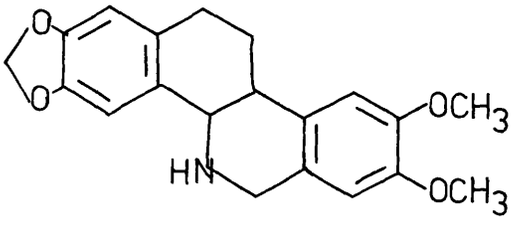


(130)

90
Also, Kametani and his co-workers have produced nitidine (9), albeit in low yield, from a benzyne reaction. The key intermediate tetralone (133) was formed by reaction of 3,4-dihydro-6,7-methylenedioxy-1(2H)naphthalenone (131) and 1-bromo-3,4-dimethoxybenzene (132) with sodium amide in refluxing dry tetrahydrofuran.

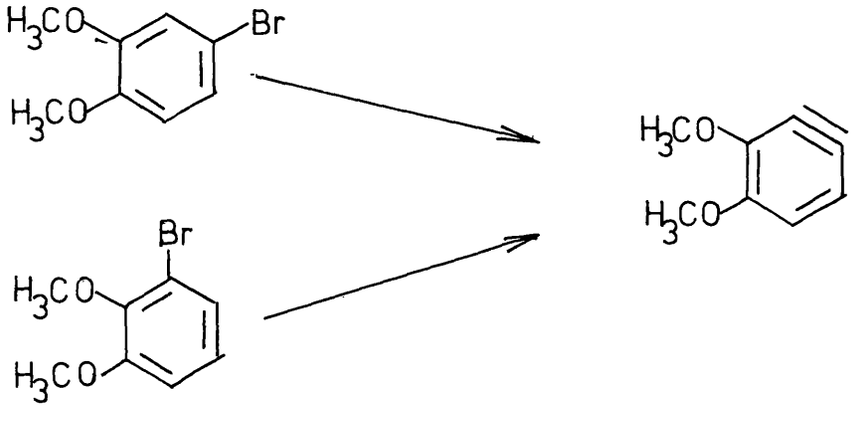


(133) was converted to the amine (134) via the oxime, and a Mannich reaction gave the benzo(c)phenanthridine (135) which was subsequently converted to the natural product.

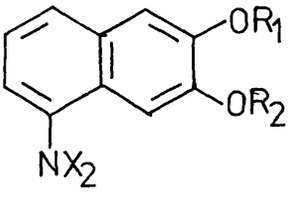


(135)

The mechanism of this type of reaction makes it unlikely to be used for unsymmetrically oxygenated analogues since the benzyne coupling will always take place <u>meta</u>- to an oxygen. This would have occurred even if, in this case, 1-bromo-2,3-dimethoxybenzene had been used.



This difficulty has been overcome by Stermitz et al.[91] in which the key intermediate is the 1-aminonaphthalene (136a),



(136)

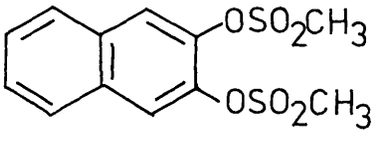
a X=H R_1=R_2=H

b X=O R_1=R_2=H

c X=H R_1=C_3H_7 R=CH_3

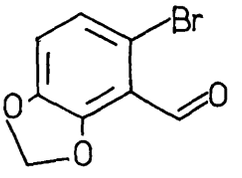
d X=H R_1=CH_3 R_2=C_3H_7

which was obtained by reduction of the nitro compound (136b) formed by acetyl nitrate nitration of the bis mesyloxy-derivative (137) of 2,3-dihydroxynaphthalene.



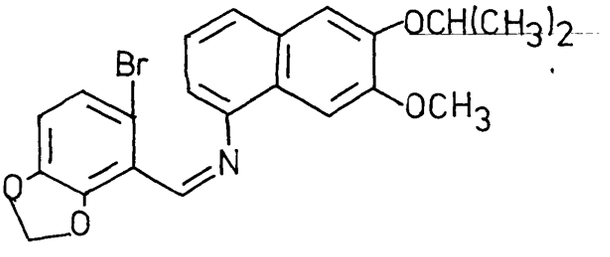
(137)

Reaction of (136c) with the bromoaldehyde (138)



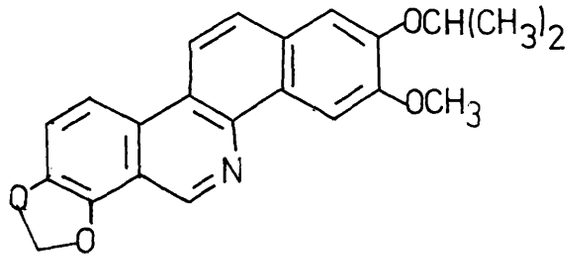
(138)

gave the anil (139)



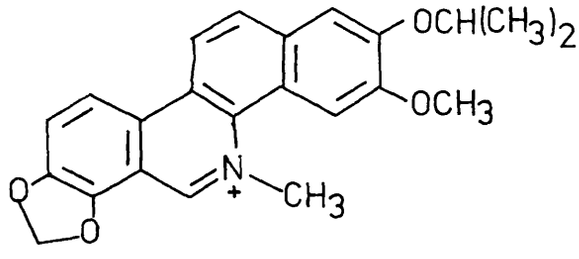
(139)

which was converted to the aromatic N-norbenzo(c)phenanthridine (140) by the action of sodamine in liquid ammonia.



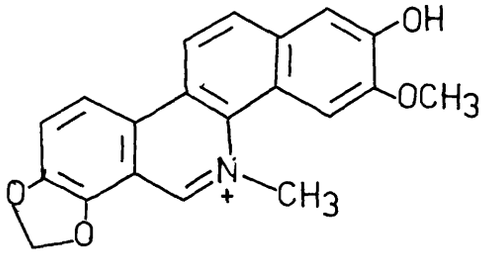
(140)

Standard aklylation methods gave the salt (141)



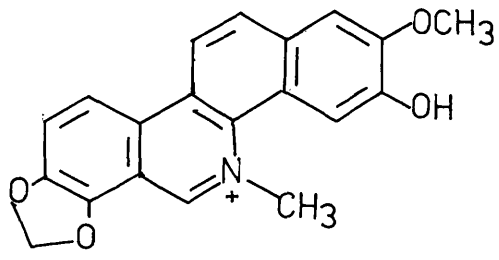
(142)

Fagaronine (142) was produced by removal of the isopropoxy protecting group by treatment with HBr/acetic acid.



(143)

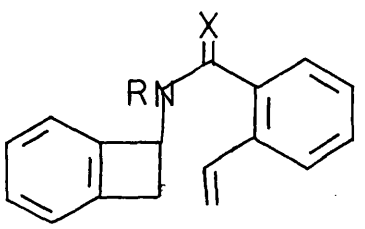
Isofagarine (143) was synthesised in the same way starting from (136d)



(143)

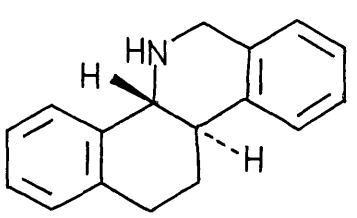
ix) Total synthesis of chelidonine

A total synthesis has been carried out by Oppolzer and Kellar[56] in which the stereoselective skeleton forming step is the thermolysis of the compound (48).



(144)

Although thermolysis of the amide (144, R=H,X=O) led to the trans fused product (145), similar treatment of the urethane (144, R=CO_2Me,X=H_2) produced the cis fused product due to greater structural flexibility (146).



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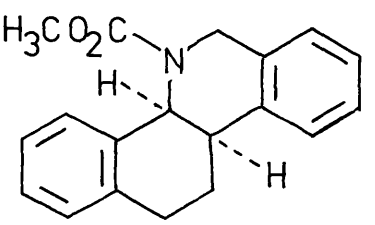
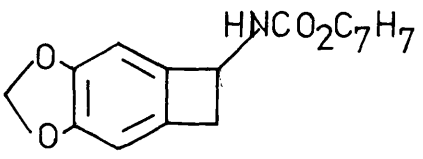
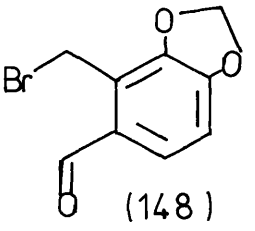


(146)

Using the simple building blocks (147) and (148)

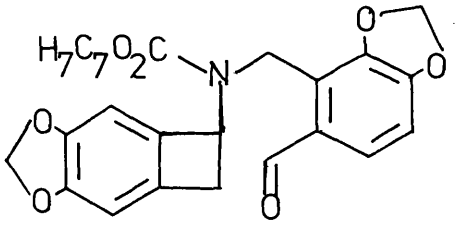


(147)



(148)

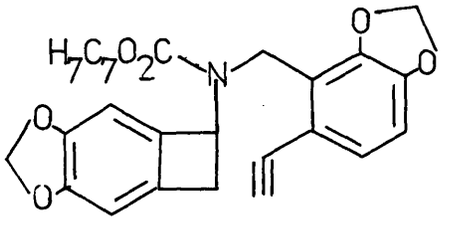
the urethane (149) was formed by reaction of the sodium salt of (147) with (148) in dimethylformamide.



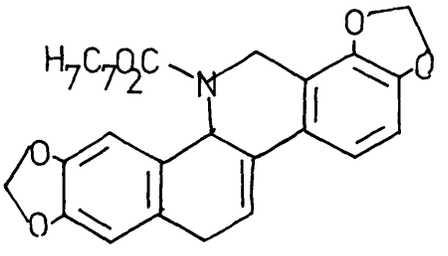
(149)

Subsequent transformations are set out below. Bromination followed by dehydrobromination gave the acetylene (150) which re-arranged to the tetrahydrobenzo(c)phenanthridine (151). Hydroboration followed by oxidation with hydrogen peroxide gave the alcohol (152) as a 1:1 mixture of epimers which were easily separated by column chromatography. Oxidation of (152) gave the ketone (153) which was stereospecifically reduced with sodium borohydride in methanoldioxane to the <u>cis, cis</u> alcohol (154). Hydrogenolysis gave dl-norchelidonine which was easily

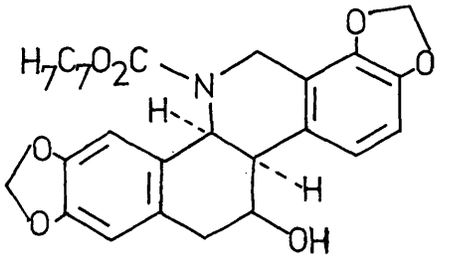
converted by N-methylation to dl.-chelidonine which was identical in all respects to the natural product.



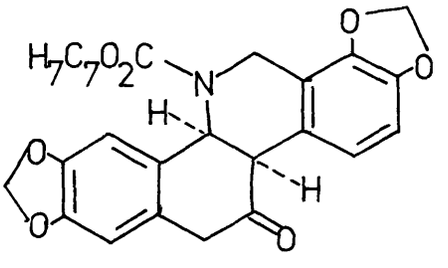
(150)



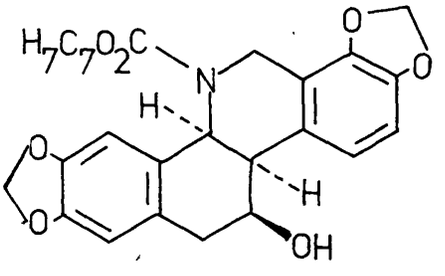
(151)



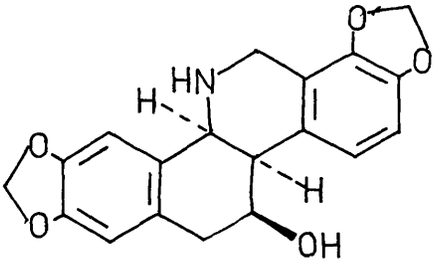
(152)



(153)



(154)



dl-norchelidonine

CHAPTER 2

INTRODUCTION

During the last century over eighty epidemics of dropsy have been recorded in India[92]; other epidemics of the disease in other tropical areas, notably the West Indies, Mauritius and tropical Africa, have been recorded during this period[93,94] although these have been apparently less severe than the Indian outbreaks. The last recorded epidemic occurred in Bombay in 1966[95] and affected several hundred people and a major epidemic of 1935 affected 7,000 people of whom 1,500 died. The vast size of India and the remoteness of some areas suggest that the number of recorded epidemics could be only a fraction of the total.

The usual symptoms of epidemic dropsy are swelling of the legs and extremities coupled with gastro-intestinal disturbances[92]. An anaemia condition can also occur[96]. Post mortem examination of most body tissue shows histopathological changes to have taken place[97]. A review of the historical data shows that a symptomless form of glaucoma was first noted in epidemic dropsy cases by Maynard in 1909[98]. The condition was characterised by:

i) increased intraocular pressure

ii) dimmed vision with haloes

iii) corneal oedema

iv) retinal haemorrhage

v) field loss unless the pressure was relieved surgically

or as a result of general recovery from the dropsy condition. There is usually no inflammation or pain. Cupping of the retina and atrophy of the optic nerve usually occur late in the course of the disease, the victim not knowing the extent of the disease until the onset of partial or total blindness.

Glaucoma

Glaucoma is normally defined as a blinding disease localised in the eyeball in which the intraocular pressure of the anterior chamber is raised considerably by either over-secretion of aqueous fluid or by blockage of the normal drainage canals. This can be by anatomical, physiological or pathological means, or a combination of all three. Its effects are usually mitigated by physically relieving the pressure by the creation of artificial drainage canals (IRIDECTOMY). Normal eye pressure is 15mm. Hg. with some diurnal variation.

Argemone glaucoma

Initial symptoms are fluctuations in eye pressure[99] of up to 70mm., later stabilising at a higher pressure. A gradual diminution of vision is also experienced.[100,101] Unlike many glaucoma conditions, this is not initially accompanied by the usual nausea, vomitting and pain: the most obvious symptom to the patient is the diminution of vision caused by atrophy[99] of the optic nerve when the disease is in its later stages and irreversible damage already done. Eyes show no inflammation,[92]

although post mortem examination of poisoned subjects revealed retinal haemorrhage and degeneration,[102-106] particularly in senile cases.

The connection between the outbreaks of "epidemic" dropsy (and its associated conditions such as glaucoma) and adulterated or contaminated vegetable oils was first noted, in India, by Sarkar[107] who discovered that all the victims of an outbreak had consumed oil from a press which had previously been used for extracting Argemone mexicana seed oil.

Since then, argemone seed oil has been implicated in most of the outbreaks where forensic investigation has been possible.

In one recent outbreak, victims were asked to provide samples of cooking oil for analysis: sanguinarine was detected[108,109] in most cases, implying adulteration or contamination by Argemone seed oil.

Sanguinarine has been detected in the blood and urine of argemone poisoning victims, both by this author (qv) and by others.[108,110]

Sanguinarine was detected[111] in the placenta of an argemone victim who aborted during the course of her illness; this is a common symptom and has been noted in humans, laboratory animals

and farm animals.[111] It is appreciated by the Ministry of Agriculture, for instance, that grazing on Chelidonium majus (greater celandine) can cause spontaneous abortion in cattle.

It should be borne in mind that due to the social and religious conventions of India the consumption of non-animal fats is very high and consequently the extraction and marketing of vegetable oils and vegetable oil products is a major commercial undertaking. At the same time, the extent of organised and intensive agriculture dictates that the cultivation of edible oil crops be almost a "cottage industry" with much of the cultivation being on smallholdings and collection being on a local and regional basis. It can be appreciated that there is plenty of scope for adulteration given a prolific weed such as Argemone mexicana with a high seed-oil content, this being up to 30% by weight of the seed. It is improbable, of course, that this adulteration is done with the connivance of the oil producing companies since the relationship between gross adulteration of argemone oil and dropsy epidemics is now well documented and accepted. The tests adopted by the oil companies are, however, so insensitive and imprecise as to suggest that they are only interested in reducing the level of contamination rather than eliminating it altogether.

The ingestion of small amounts of sanguinarine over long periods of time has been suggested by Hakim[112] as a possible cause

of endemic glaucoma as opposed to the epidemic form mentioned above. Endemic glaucoma is a disease of enormous world wide proportions. In the United Kingdom, one per cent of the population over the age of 40 years is a glaucoma victim and a large proportion of these cases result in total blindness.[113]

Similar statistics apply in most developed countries. The situation in the undeveloped world is, of course, open to speculation although since some protection is obtained from a high-protein diet one would expect the position to be much worse.

Hakim suggests several ways in which sanguinarine can be innocently ingested over long periods of time. Obviously, edible oil products with low level contamination is an obvious choice in countries where this is possible.

[114] Hakim has shown that ingestion of the foliage of Argemone mexicana led to the presence of sanguinarine in the milk of goats and cattle. This also occurred when the animals were fed with oil-seed cake containing argemone seed. Sanguinarine was also concentrated in the flesh (particularly the liver) of animals which had been allowed to feed on A.mexicana foliage.

Studies with poultry showed that when hens were fed on corn containing argemone seed,sanguinarine was detected in the eggs.

Feeding of the contaminated milk or eggs caused rises in intraocular pressure in experimental animals. It is quite possible[115] that ingestion of contaminated dairy produce over a long period of time could lead to glaucoma in later life.

It may be objected that sanguinarine is a rare alkaloid unlikely to appear in a Western diet and until recently this was believed to be the case. It now appears, however, that sanguinarine is an extremely common alkaloid and some screening work by Hakim, Mijovic and Walker[112] indicates that it is probably present, although in small quantities, in most of the 675 species of Papaveraceae, a plant family which, of course, has world wide distribution.

Table 7 shows the occurrence of sanguinarine and other benzo(c)phenanthridines in species of Papaveraceae found in the United Kingdom. It is true, of course, that where good pasture exists animals would tend not to feed on papaveraceous plants but in certain areas of the world this is not possible due to the preponderance of plants of this family.

Other routes to ingestion exist[116]: Argemone foliage is eaten as a salad vegetable in parts of India and Iran (as is the foliage of Papaver somniferum in areas where this plant is cultivated). Argemone roots are used to prepare a fermented beverage in parts of Africa and Argemone oil is used as a

purgative in Africa and India.

Sanguinarine can be directly ingested by the use of culinary poppy seed, which have been found by several authors including this author to contain sanguinarine, which is often sprinkled on bread or incorporated into poppy seed cake. Some cultural groups, for example the Jewish community, make considerable use of poppy seeds in cooking and a higher than usual incidence of glaucoma has been noted for these groups.[117]

Of most relevance to the developed world is the ingestion of sanguinarine through contaminated dairy and meat products. Some preliminary work in correlating glaucoma distribution with the geographical distribution of papaveraceae and of climatic conditions has been carried out.[108]

TABLE 7

BRITISH PAPAVERACEAE

ABUNDANCE	PLANT SPECIES	CITATION	BENZO(C)PHENANTHRIDINE											
			1-norchelidonine	oxysanguinarine	sanguinarine	dihydrosanguinarine	oxychelidonine	chelidonine	homochelidonine	methoxychelidonine	chelerythrine	chelilutine	chelirubine
	PAPAVER												
xx	P.argemone Linn.	FAB		x									
vr	P.atlanticum Ball	F			x								
xxx	P.dubium Linn.	FAB		x	x								
x	P.hybridum Linn	FAB		x	x								
xxxx	P.rhoeas Linn.	FAB		x	x						x		
x	P.somniferum Linn.	FAB			x								
	FUMARIA												
x	F.bastardii Boreau	FA											
x	F.capreolata Linn.	FAB											
x	F.densiflora DC	F											
vr	F.martinii Clavaud	FA											
x	F.micracantha Lag.	B			x								
xx	F.muralis												
	ss muralis Koch	FAB											
	ss boraei Pugs.	F											
	ss neglecta Pugs.	F											
xxxx	F.officinalis Linn.	FAB			x								
x	F.parviflora Lam.	FAB											
x	F.purpurea Pugs.	FA											
x	F.vaillantii Loisel	FA			x								
x	F.occidentalis Pugs.	FA											
	OTHERS												
vr	Argemone mexicana Linn.	F			x	x	x				x		
xxxx	Chelidonium majus Linn.	FAB		x	x		x	x	x	x	x	x	x
xx	Corydalis lutea Linn.	FAB			x								
xx	C.claviculata Linn.	FAB											
x	Glaucium flavum Crantz	FAB	x		x			x					x
vr	G.corniculatum Linn.	F			x			x			x		x
x	Eschscholtzia californica Cham.	F			x						x	x	x
x	Meconopsis cambrica Linn.	FAB			x						x		

KEY TO TABLE OF BRITISH PAPAVERACEAE

Citations

The plant species listed are cited in at least one of the three major Floras:

F = Flora Europaea	Cambridge University Press
A = Atlas of British Flora	Ed. Perring & Walters
B = British Flora	Bentham & Hooker

Some differences of classification occur:

F.vaillantii Loisel = F.parviflora Linn. in 'British Flora'

F.muralis ss muralis Koch = F.capreolata Linn. in 'British Flora'

F.micracantha Lag. = F.densiflora DC in 'Flora Europaea'

P.dubium Linn. = P.lecoqii Lam. (Index Kewensis)

Abundance

Abundance is classified as follows:

xxxx	very common
x	rare
vr	very rare

PHARMACOLOGY OF SANGUINARINE

A considerable amount of work on the pharmacology of the seed oil of Argemone mexicana Linn. and sanguinarine was carried out by Hakim,[92] corroborating and developing the earlier studies of Meyer.[119] His findings can be summarised thus:

i) sanguinarine depresses the actions of sympathetic stimulation and of adrenaline

ii) sanguinarine stimulates, then subsequently depresses, adrenaline-like actions

iii) adrenaline reduces or abolishes the action of sanguinarine

iv) the action of acetylcholine is decreased by sanguinarine.

In the same paper Hakim describes some biochemical effects of sanguinarine. In a series of experiments he found that

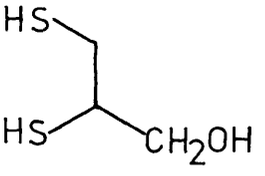
i) sanguinarine enhanced the action of insulin

ii) sanguinarine did not cause a significant increase in blood pyruvate levels in rats

iii) sanguinarine inhibits cholineacetylase in in-vitro experiments.

The fact that sanguinarine does not cause a significant increase in blood pyruvate levels was unexpected, since blood pyruvate levels were known to be higher than normal in Argemone

oil dropsy cases and it has been shown[120] that sanguinarine is toxic to several physiological enzyme systems including pyruvate oxidase. Hakim rationalises this by assuming pyruvate oxidase blockage to be a symptom of high-dose acute poisoning, although no evidence is offered. Previously, Sarkar[120] had found that sanguinarine chloride inhibited the <u>in-vivo</u> oxidation of pyruvate, succinate and lactate and suggested that the inhibitory effect of sanguinarine is due to its action on -SH enzyme systems. This theory is substantiated by the observation that although BAL (British Anti Lewisite, 2,3-dimercaptopropanol) (155) would not reverse the effects of sanguinarine; pretreatment with BAL gave effective treatment. Further evidence is provided by the observation that epidemic dropsy generally affects populations with low protein diets[121], particularly where the intake of cysteine is low. Goats fed argemone oil incorporated in a low cysteine diet were affected to a greater extent than those where the Argemone oil was incorporated in a normal diet.[122]



(155)

In an experiment to investigate the effect of Sanguinarine on the drainage mechanism of the anterior chamber of the eye Hakim perfused the anterior chamber of an isolated ox eye with an artificially compounded ox aqueous fluid through a fine bore needle. Addition of a dye to the perfusing fluid indicated normal drainage through the filtration angle. Addition of sanguinarine chloride or Argemone seed oil immediately stopped the outflow of dyed aqueous fluid for periods of up to thirty minutes. Direct injection into the anterior chambers of the eyes to live animals had the same effect, as shown by a rise in intraocular pressure. Subsequently this phenomenon was demonstrated with chelerythrine (7) and with oils and latexes from several other species of Papaveraceae.

The work was taken further by Liebe and Scherf[123], who found that intravenous injections of other isoquinoline alkaloids produced similar acute rises in intraocular pressure. The isoquinolines found to exert this effect are shown in Table 8.

TABLE 8

Isoquinoline alkaloids causing increased intraocular tension[123]

Papaverine	∝-allocryptopine *
(±)-salsolidine	biculline
laudanosine	narcotine
berberine *	cularine
coptisine *	chelerythrine *
corydaline	morphine
tetrahydropalmitine	codeine
ophiocarpine	thebaine
isocorydaline	narceine
bulbocapnine	glaucine
chelidonine	corybulbine

*Alkaloids reported in A.mexicana Linn.

In an elegant development of his work, Hakim[124] produced increases in intraocular pressure by direct stimulation of the hypothalamus area of the brain with sanguinarine. This approach had been suggested by the work of Sallman and Lowenstein[125] and of Gloster and Greaves[126] who had produced increases in intraocular pressure by electrical stimulation of the hypothalamus.

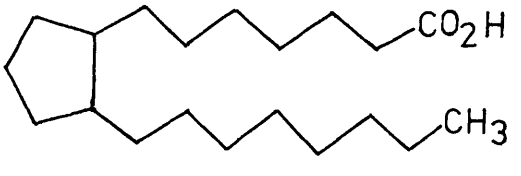
It appeared likely that a neurohormone, whose release could be triggered either by pharmacological or electrical means, was responsible for the increase in intraocular pressure.

Hakim reasoned that a neurohormone released into the eye of an experimental animal could be used to stimulate increases in intraocular pressure in a second animal. An experiment was designed[115] which was similar in approach to those described by Bain[127] and by Loewi.[128] In these classical experiments, two isolated, beating, frog hearts were perfused in series; stimulation of the vagus nerve of the first heart led to the release of acetylcholine which slowed the beating of both stimulated and recipient hearts. Hakim perfused the left eyes (in situ) of two cats, in series, with saline.

The cornea of the first eye was pierced by a specially designed needle which permitted fluid to be continuously introduced to, and removed from, the anterior chamber after which it was dripped continuously onto the eye of the second

(anaesthetised) cat. Direct stimulation of the hypothalamus of the first cat, by introduction of sanguinarine chloride via a cannula, led to an increase in intraocular pressure in the second cat. The other eye of the recipient cat which was constantly dripped with saline as a control was not affected. Similar results were obtained when sanguinarine chloride was introduced to the donor cat as an intravenous drip.

Hakim postulates the existence of a ciliary-iris hormone, for which he proposes the name 'occulo-tensin', which controls the aqueous pressure in the eye. He suggests that this hormone may be a prostaglandin (157) and that prostaglandin $F_2\alpha$(158) is the most likely candidate.



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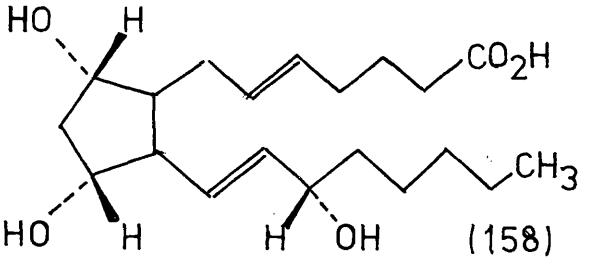


(158)

The evidence for this is largely circumstantial;

i) prostaglandins are components of the extracts of iris material: Ambache[129-132] extracted a mixture of prostaglandins E_2 and $F_2\alpha$ from the rabbit iris and also from the cat iris. Anggard and Samuelsson[133] have demonstrated the presence of protaglandin $F_2\alpha$ in the sheep iris

ii) the effect of 'occulo-tensin' was abolished[134] by the enzyme 15-hydroxyprostaglandin-dehydrogenase in 'double-cat' experiments (cf ref 103)

iii) the effect of 'occulo-tensin' was abolished[135] by aspirin: this is a property typical of prostaglandins

iv) prostaglandin $F_{2\alpha}$ is known[136-137] to have the effect of producing prolonged constriction of veins (in this case the effect would be on the subconjunctival aqueous veins which are part of the eye's drainage mechanism).

Hakim[135] postulated a continuous secretion of prostaglandin $F_{2\alpha}$ in the eye in order to maintain constrictive tone in the drainage channels (thus maintaining a certain amount of necessary aqueous pressure in the tye). This is supported by the fact that aspirin, an acknowledged prostaglandin antagonist, lowers[135] the pressure in the normal eye.

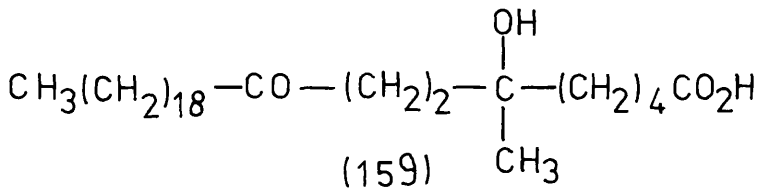
The release of hormone is controlled by the hypothalamus: this explains the diurnal variation in eye tension in the normal eye and the fact that one of the initial symptoms of Argemone glaucoma is an increase in the range of the normal diurnal fluctuation, a consistent, permanent rise in eye pressure being a later development.

Pharmacological and toxicological studies in several species of animal noted[138] a difference in biological activity between sanguinarine chloride and Argemone mexicana seed oil, the seed oil being several times more active than could be explained by its apparent sanguinarine content.

Sanguinarine chloride, administered intravenously or orally in doses lethal to rats was found[138a] to have no effect on rhesus monkeys although argemone mexicana seed oil in doses *pro rata* produced oedema.[138b] Similar amounts of argemone oil from which the alkaloids had been removed showed no biological effects. Furthermore, the effect of sanguinarine chloride appeared to be transitory in many cases. It appeared likely to some workers[139,140] that another compound was present in the seed oil, a potentiating factor, which, in combination with sanguinarine exerted the biological effect.

In investigations by Rukmini[140] a solid fatty acid was isolated by extraction of the oil with petroleium ether. The compound was found to be non-toxic as was the extracted argemone oil. However, when the two fractions were recombined or when sanguinarine chloride was combined with the fatty acid, normal argemone toxicity was again observed.

Chemical and spectroscopic examination[141] suggest the compound to be (+)-6-hydroxy-6-methyl-9-oxo-octacosanoic acid (159).



(159)

(159a)

Elemental analysis coupled with mass spectroscopy showed the composition to be $C_{29}H_{56}O_4$. The compound formed a methyl ester and an oxime and was optically active. The IR spectrum of the methyl ester showed the presence of a ketocarbonyl group (ν_{max} 1738cms-1); acetylation did not occur with pyridine/acetic anhydride but a mono-acetate was formed with perchloric acid/ acetic anhydride, indicating a sterically hindered hydroxyl group. Reduction with $LiAlH_4$ produced a trihydroxy compound which in turn gave a triacetate with perchloric acid/acetic anhydride. Beckman rearrangement of the oxime followed by hydrolysis led to the recovery of the amine $CH_3(CH_2)_{18}NH_2$ and an unsaturated dicarboxylic acid which gave isosebacic acid (159a) on hydrogenation; this indicated that the carbonyl group was situated at C-9, subsequently confirmed by the appearance of a MacLafferty rearrangement fragment in the mass spectrum.

Dehydration of the methyl ester followed by oxidation with permanganate/periodate led to the isolation of glutaric acid,

which suggested that the hydroxyl group was situated at C-6.

It would appear from these observations that sanguinarine chloride requires the presence of a second compound before its full biological effect can be observed. Confirmation of these findings in well designed cross-over experiments in selected animals is required. In the absence of confirmation it may be that the difference in toxicity between argemoneoil and sanguinarine is apparent rather than real - if the dose is calculated on the amount of quarternary base extracted from the oil by conventional means then this could indeed be several times less than the amount of sanguinarine (as the pseudo base) actually present in the seed oil. Many workers including Hakim have assumed that the dihydrosanguinarine isolated from the seed oil is a bona fide constituent and not an artefact of isolation; since dihydrosanguinarine has been found to be non-toxic 80-90% of the sanguinarine present in the oil may have been ignored. Another significant fact is that while all the toxicology and pharmacology of sanguinarine has been carried out on the quarternary salt, usually the chloride, in the real situation the alkaloid is presented to the system as the tertiary (pseudo) base and this raises several questions as to the validity of any simple comparisons between argemone oil and sanguinarine chloride in in-vivo situations. Comparisons using oral administrations are particularly suspect.

ANALYTICAL DETERMINATION OF SANGUINARINE

Since the involvement of Argemone oil and sanguinarine in epidemic dropsy was well established and its role in endemic, chronic glaucoma supported by a growing body of circumstantial evidence an analytical method was required that could detect and estimate sanguinarine in low concentrations in edible oils, food products and physiological fluids and tissue.

As it was hoped that the final estimation method would be used as a routine screening procedure for suspect foodstuffs and for suspected cases of argemone poisoning it was essential that the method be simple, rapid, and use the minimum of sophisticated equipment. A further important consideration was that the procedure would probably need to be carried out by semi-skilled personnel.

Several methods exist for the detection of adulterated edible oils. Those recommended by the Indian authorities are simply qualitative procedures which indicate the presence of sanguinarine but not the extent of the contamination. In one procedure[142] a sample of the oil is treated with hydrochloric acid and ferric chloride; where sanguinarine is present red/brown crystals appear at the interface. In an alternative test[143] a sample of oil is treated with hydrochloric acid: sanguinarine is extracted into the acid aqueous phase and imparts a red coloration.

Neither procedure gives a conclusive result when the concentration of argemone oil is less than 1% and neither test is

a) quantitative

or

b) specific for sanguinarine.

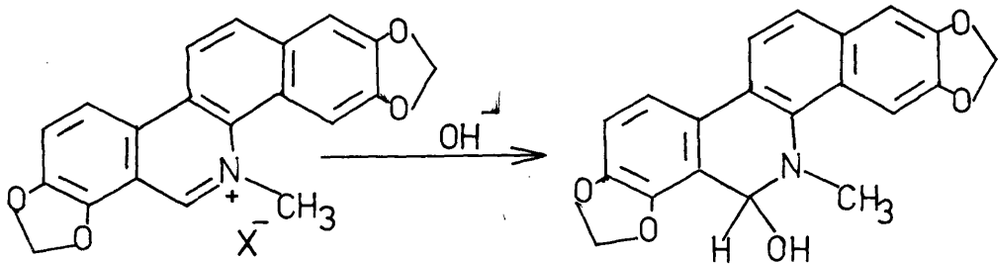
Analytical procedures involving paper chromatography have been described by Hakim[112,114,144] and by Chakravarti et al[145], and Hakim[146] has also described an electrophorelic technique. A method employing thin layer chromatography of the gross oil sample has been described by Verma et al.[147] All the methods above are simply detection procedures which give no quantitative estimation of alkaloid content.

A colorimetric procedure described by Bose[148], in which the colour developed by addition of antimony trichloride is measured, claims to be quantitative but has several defects,not least of which is that at no point in the procedure is a solution diluted to standard volume!

It can be said, therefore, that the existing methods are nonquantitative or, at best, semiquantitative.

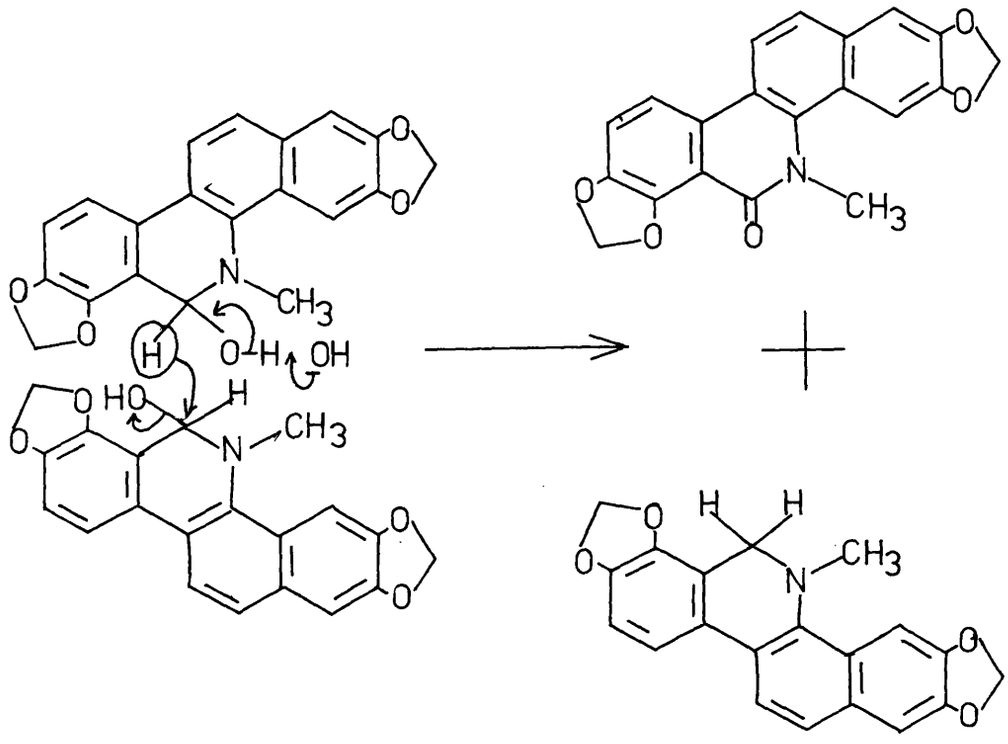
The main problem encountered in the analysis of sanguinarine is in the initial extraction of the alkaloid.

Phytochemical investigations carried out both by this author and by others have shown that 95-100 per cent of the alkaloidal material obtained from the seed oil of Argemone mexicana is a mixture of sanguinarine (6) and dihydrosanguinarine (31) the proportions are variable but dihydrosanguinarine predominates, being 80-90 per cent of the mixture. Dihydrosanguinarine has been found by Hakim and Sewkar to be non toxic and so at first sight it would appear that the toxic principle sanguinarine is a relatively low (10-20 per cent) fraction of the total alkaloids present. It is generally accepted, however, that dihydrosanguinarine is an artefact of the extraction procedure, produced by the disporportionation of the pseudo base of sanguinarine (5). This reaction is known to occur <u>in-vitro</u> when sanguinarine salts are treated with aqueous base:



(5)

The disproportionation reaction then proceeds, two molecules of the pseudo base producing one molecule of oxysanguinarine and one of dihydrosanguinarine. It is conceivable that this is an intermolecular reaction involving transfer of hydrogen with its electron pair.



This would have to occur in a concerted manner in a bimolecular complex since migration of free hydride ions is unlikely in aqueous media. It can be seen that this type of reaction could take placenot only during the later stages of a classical base work-up procedure but also in the early stages since sanguinarine is present in the plant as a pseudobase - sugar conjugate and not as the quarternary salt. Initial hydrolysis of the pseudobase-sugar conjugate could also lead to disproportionation products.

Thus, any analysis procedure must be capable of either extracting and assaying sanguinarine plus the sanguinarine derived products dihydrosanguinarine and oxysanguinarine which would be very difficult, or of transforming the sanguinarine *in situ* into a compound with properties suitable for extraction and estimation.

It was realised relatively early on that direct extraction and estimation of sanguinarine based alkaloids was not a practical proposition. It was decided, therefore, to modify the sanguinarine *in situ* and avoid interference by artefacts.

It was thought that the simplest way to facilitate total extraction of the alkaloid and to improve its stability during analytical manipulation was to reduce the alkaloid to the 5,6-dihydroderivative, a simple reaction using sodium borohydride[149]. Complete removal of the tertiary base would then be possible using simple extractive techniques.

Initially the oil was treated with a solution of sodium borohydride in ethanol, with rapid shaking of the resulting two phase mixture. After the reaction was complete, ether was added, and the mixture extracted with acid. Normal base work-up led to the recovery of dihydrosanguinarine. Unfortunately this method gave results which were very variable, indicating a problem due to the non-homogeneous nature of the reaction medium.

In order to homogenise the reaction medium it was necessary to choose a solvent which dissolved argemone seed oil and at the same time fulfilled the conditions for reduction with sodium borohydride, that is, the solvent must be capable of releasing protons. This meant that the search be confined to the lower alcohols and it was found that n-propanol was the most suitable. A solution of argemone oil in n-propanol was treated with a solution ofsodium borohydride in n-propanol. After reaction the n-propanol was removed under reduced pressure, the residue dissolved in ether and worked-up for bases. This method gave higher mean recoveries than obtained previously but still a considerable amount of variation occurred and it was felt that this was due to incomplete dissolution of the residue prior to extraction or to reaction with the n-propanol during its removal since relatively high temperatures were required for complete removal.

It was then realised that ethanol was slightly soluble in petroleum ether (40^{o}-60^{o}). It was possible, then, to produce a "solution" of sodium borohydride in ethanolic petroleum ether. Since seed oils are also soluble in petroleum ether this would be expected to give a homogeneous reaction medium and this was found to be the case.

Two methods of analysis of the extracted alkaloid were actively pursued, the first being a gas chromatographic method

by which it was hoped to simultaneously detect sanguinarine (as a derivative) and benz(c)acridine, its proposed metabolite (q.v.). This was found to be unsuitable for sanguinarine but provided a rapid analysis for benz(c)acridine, and is described in Chapter 3.

The thin layer chromatographic method eventually developed combines a rapid screening technique, non-quantitative detection down to very low levels and an accurate and precise determination within defined limits. The method can be applied to oil samples, tissue samples and physiological samples such as milk, urine, and blood (after concentration). With some initial pre-treatment foodstuffs could also be examined.

Summary

The method consists of the reduction of sanguinarine by sodium borohydride to 5,6-dihydrosanguinarine followed by extraction into aqueous acid media; basification and extraction with chloroform is followed by concentration to standard volume. Samples are applied to a thin layer (250μ) of silica gel G by either a microsyringe or precision microcapilliary and, after presaturation, developed over 10cm with a mixture of benzene and methanol. Irradiation of the air-dried plate with long wavelength untra-violet light caused reoxidation to the fully aromatic benzo(c)phenanthridine.

After removal of the tlc spot using a specially designed

micro 'vacuum cleaner' the sanguinarine was eluted from the silica gel absorbant with 95% ethanol. After making up to standard volume with 95% ethanol the absorbance at 330nm was measured. Comparison of the absorbance of the test solution with a standard curve allows the calculation of the amount of sanguinarine in the original sample.

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DISCUSSION

i) Extraction

The processes leading up to the choice of the petroleum spirit/ethanol system have already been described. The extraction data for the three methods

a) Ethanol/oil (two phase)

b) n-propanol/oil (solution)

c) petroleum spirit/oil (solution)

are shown graphically, in the form of scatter diagrams in Figures 1, 2 and 3. It can be seen that the amount of variation for the ethanol and n-propanol systems is unacceptable. Figure 3, however, shows the method of choice to give good reproduceable extraction of $95 \pm 3\%$. For data see Table 10.

Interference by other alkaloids

Several other alkaloids occur with sanguinarine in the seed oil of Argemonemexicana. Three of these have been identified as

berberine

protopine

chelerythrine

A further minor alkaloid has been tentatively identified as N-norsanguinarine. Two other minor alkaloids occur in the oil but these remain to be identified. Of the four alkaloids mentioned only chelerythrine interferes in the analysis in that

FIGURE 1

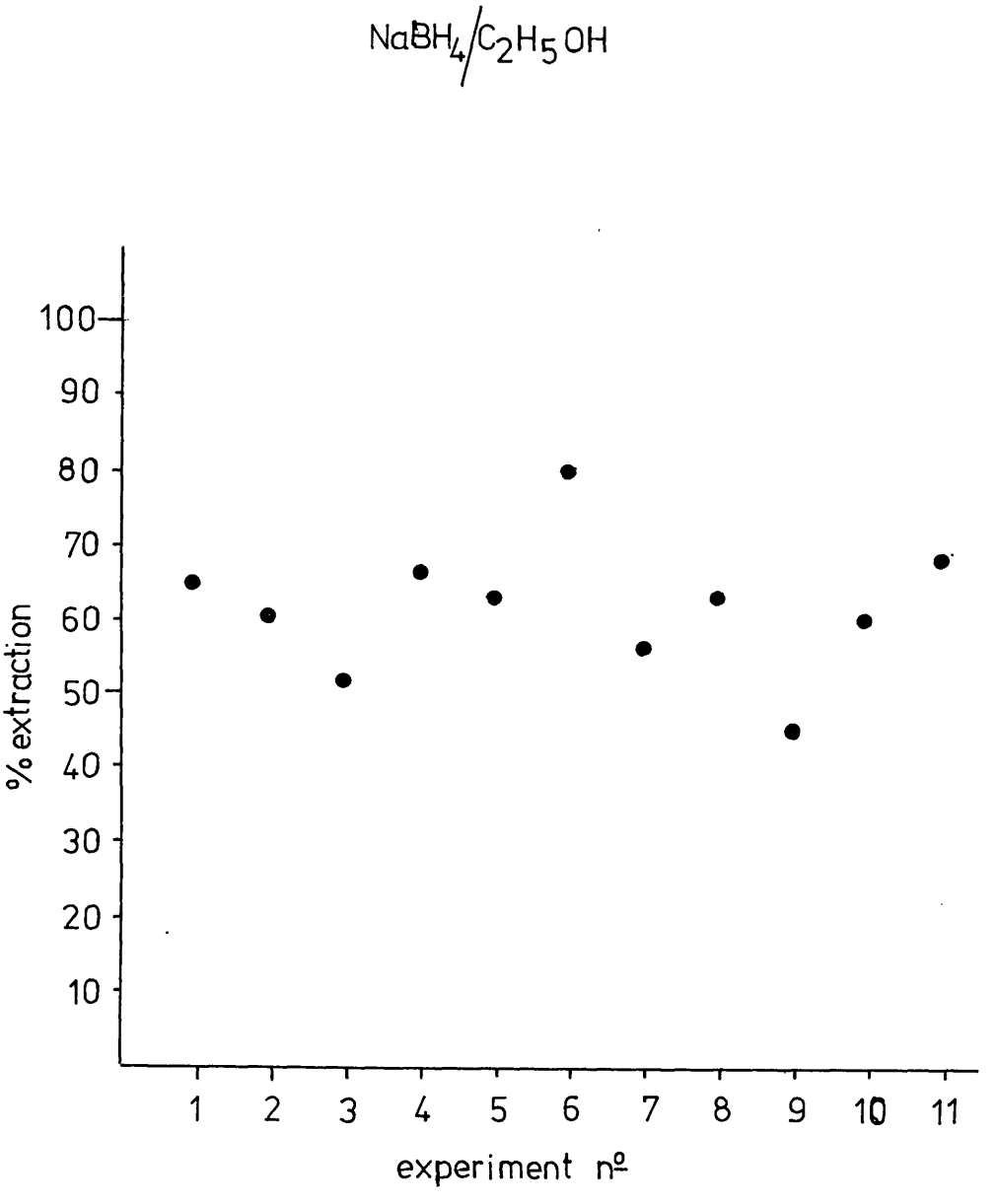

NaBH4/C2H5OH
100
90
80
70
60
50
40
30
20
10
% extraction
1
2
3
4
5
6
7
8
9
10
11
experiment nº

FIGURE 2

$NaBH_4$ / n-propanol

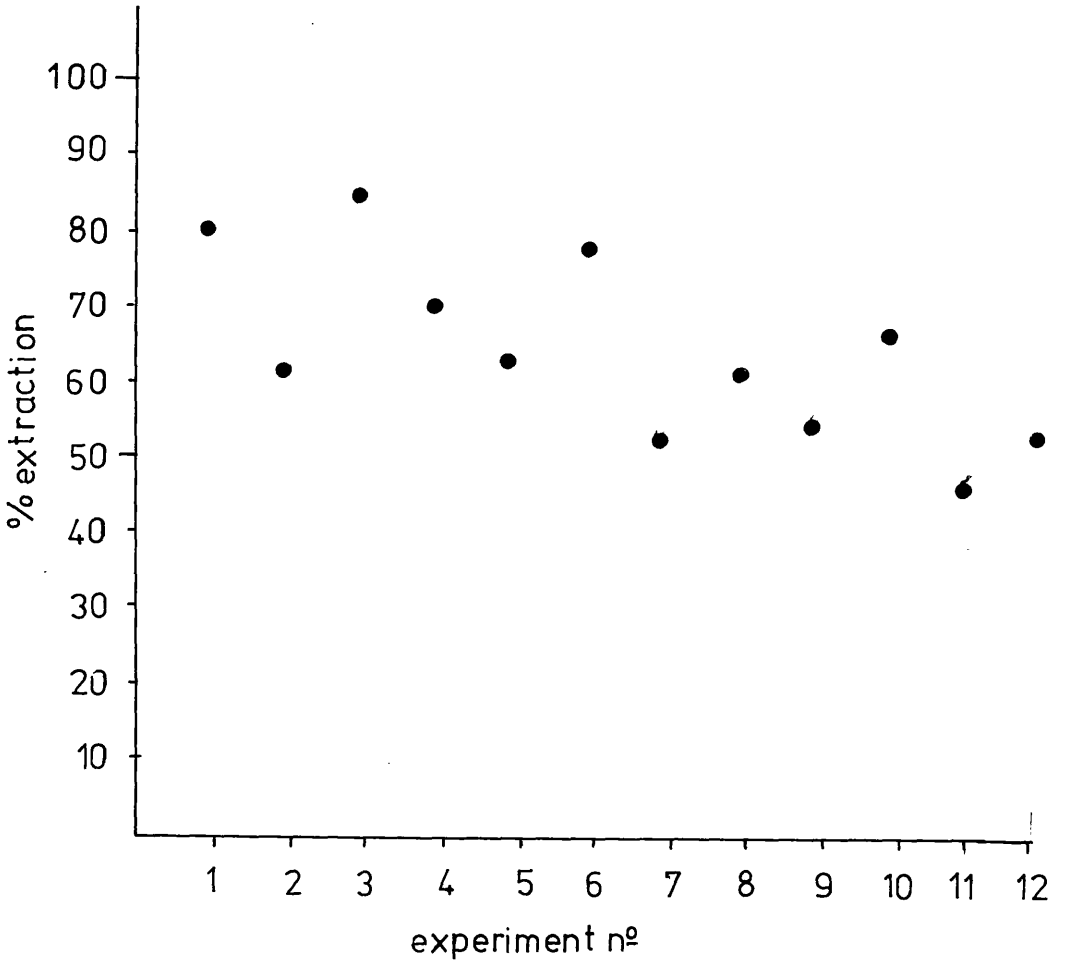


FIGURE 3

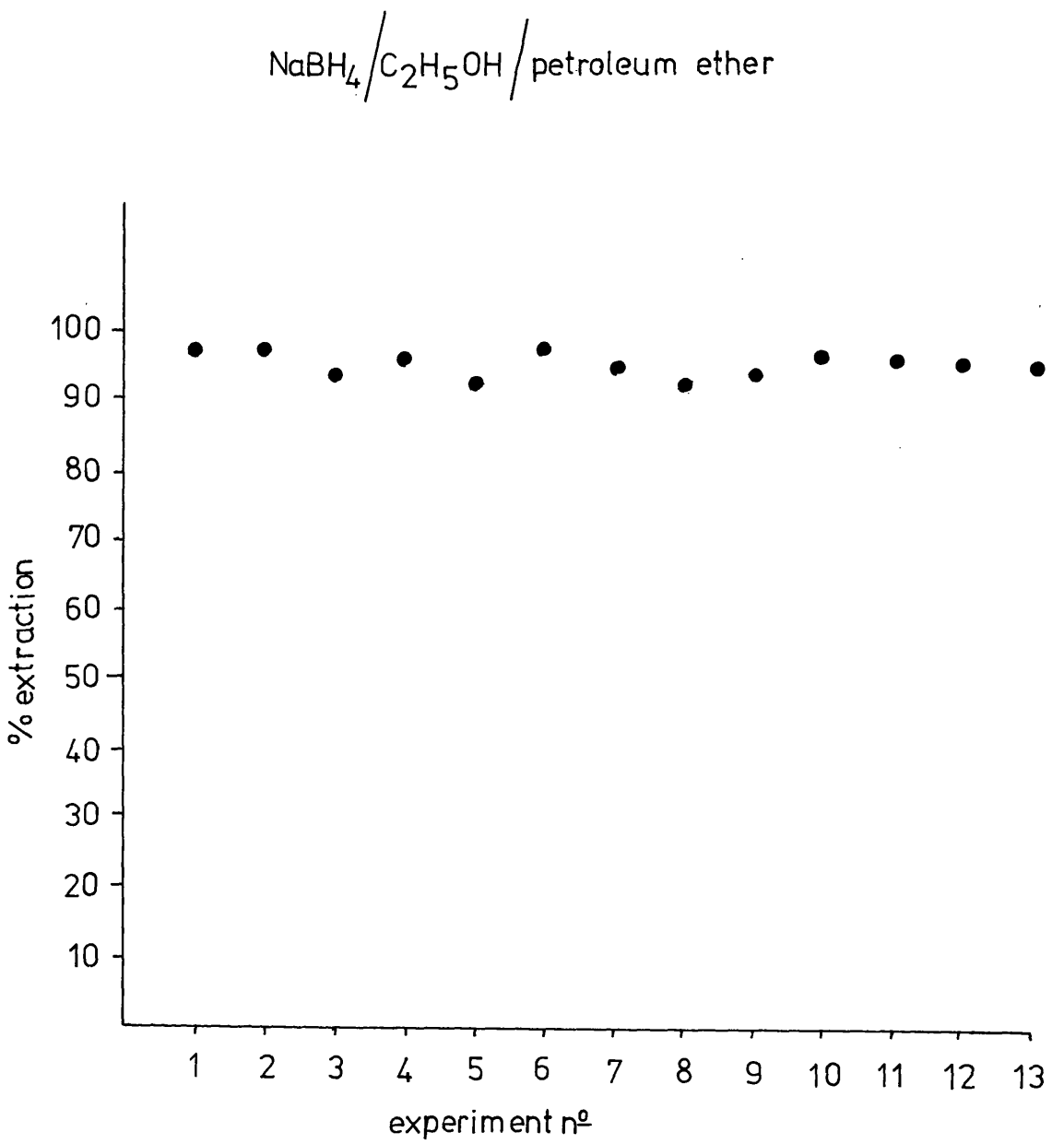

NaBH4/C2H5OH/petroleum ether
100
90
80
70
60
50
40
30
20
10
% extraction
1
2
3
4
5
6
7
8
9
10
11
12
13
experiment nº

TABLE 10

% sanguinarine extracted			
method / expt.	(a)	(b)	(c)
1	65·0	80·0	97·0
2	60·0	61·0	97·5
3	51·0	85·0	94·0
4	66·5	70·0	96·0
5	63·0	63·0	93·0
6	80·0	78·0	97·5
7	56·0	53·0	94·5
8	63·0	61·0	92·5
9	45·0	55·0	93·75
10	60·0	66·0	96·0
11	68·0	47·0	95·5
12	—	53·0	95·0
13	—	—	94·5
MEAN	61·59	64·33	95·13
$\sigma_{(n-1)}$	9·18	11·92	1·63

the resolution of the two t.l.c. spots is not complete. In the particular case of Argemone mexicana seed oil this is not important due to the difference in relative amounts present. In other situations where the disparity is not so great there may well be difficulties since the difference between the two compounds is so small as to make total separation of the two extremely difficult.

In practical terms the separation would probably be impossible using the t.l.c. techniques but, since there is a slight difference in R_f value, quantitative separation and estimation of sanguinarine and chelerythrine may be possible using High Pressure Liquid Chromatography. It may, of course, be said that it is unnecessary to separate the two since their chemical and structural similarity would imply that both have the same physiological effects.

ii) Chromatography

Chromatographic conditions were established after considering several different solvent combinations. The use of aluminium oxide as adsorbant was soon discounted due to the pseudobase transformations which were found to be taking place, and silica gel adsorbant chosen. The best separation was found to occur using a mixture of benzene and methanol in the proportion 6:1, although Slavic[5] and his group and Stermitz[15] have done a considerable amount of identification work with benzo(c)-phenanthridines using a 3:2 mixture. R_f was found to be very

dependent on the composition of the developing solvent mixture and it was also found that the adsorbant preferentially absorbed methanol. The result being that after a certain number of plates had been run the solvent mixture became depleted in methanol causing a drop in R_f value. This problem was minimised by regular monitoring of solvent compositions.

The variation of sanguinarine R_f with solvent composition is shown below (Figure 4).

FIGURE 4

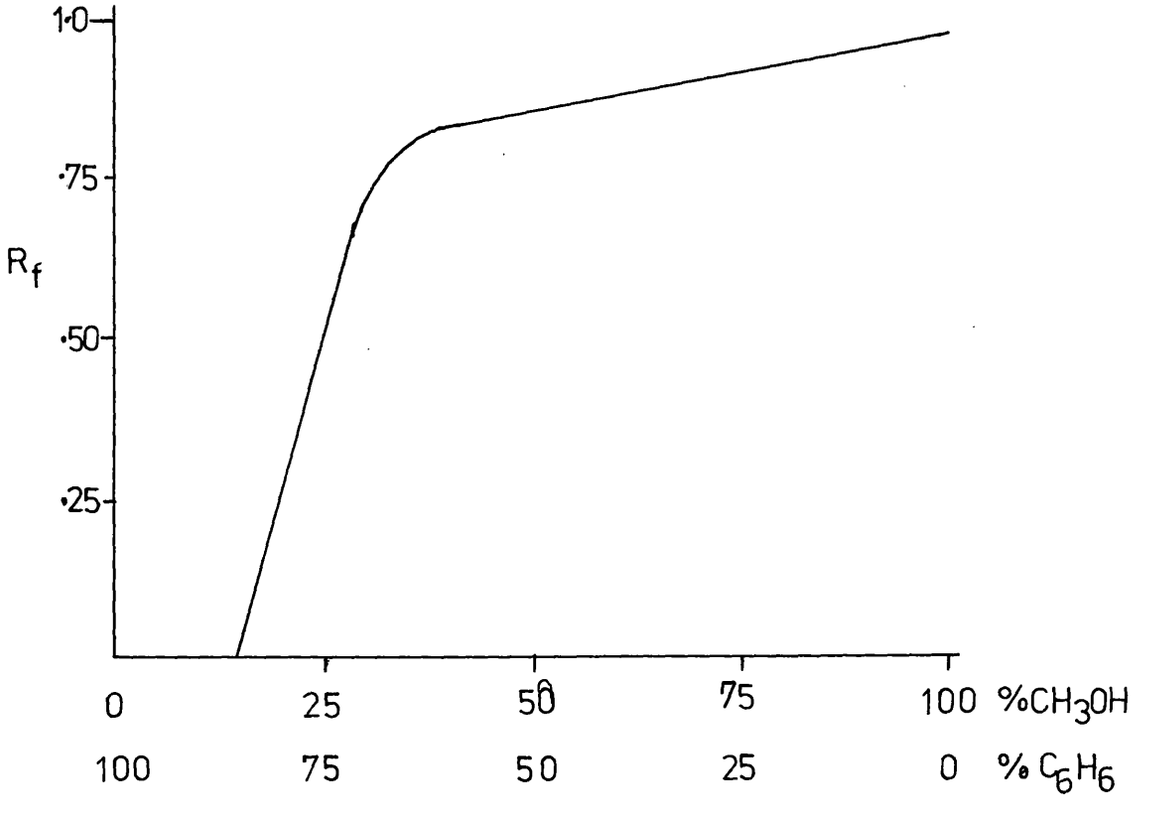


TABLE 11

REPRODUCEABILITY OF R_f

	no presaturation		presaturation[a]	
	MEAN[b]	σ	MEAN[b]	σ
TANK 1	0·770	·021	0·782	·013
" 2	0·785	·034	0·790	·020
" 3	0·777	·024	0·785	·016
" 4	0·790	·029	0·790	·016
" 5	0·776	·022	0·780	·015
MEAN	0·780	·026	0·785	·016

a 0·5 hrs. over benzene/methanol

b 20 samples/plates

Comparison of the two sets of data in Table 11 shows that the reproduceability of the R_f of sanguinarine was greatly increased by presaturation of the plates (after application of the sample spots) in an atmosphere of benzene and methanol. This procedure also reduced the amount of methanol depletion of the solvent bath.

iii) Reoxidation

Reoxidation was found to be complete after fifteen minutes of ultra-violet irradiation. This reaction has also been investigated by Stipanovic, Bell and Howell[150], who propose a free-radical reaction involving the free-radical (160) and the 6-peroxy- derivative (161) formed by reaction of the radical (160) with molecular oxygen.

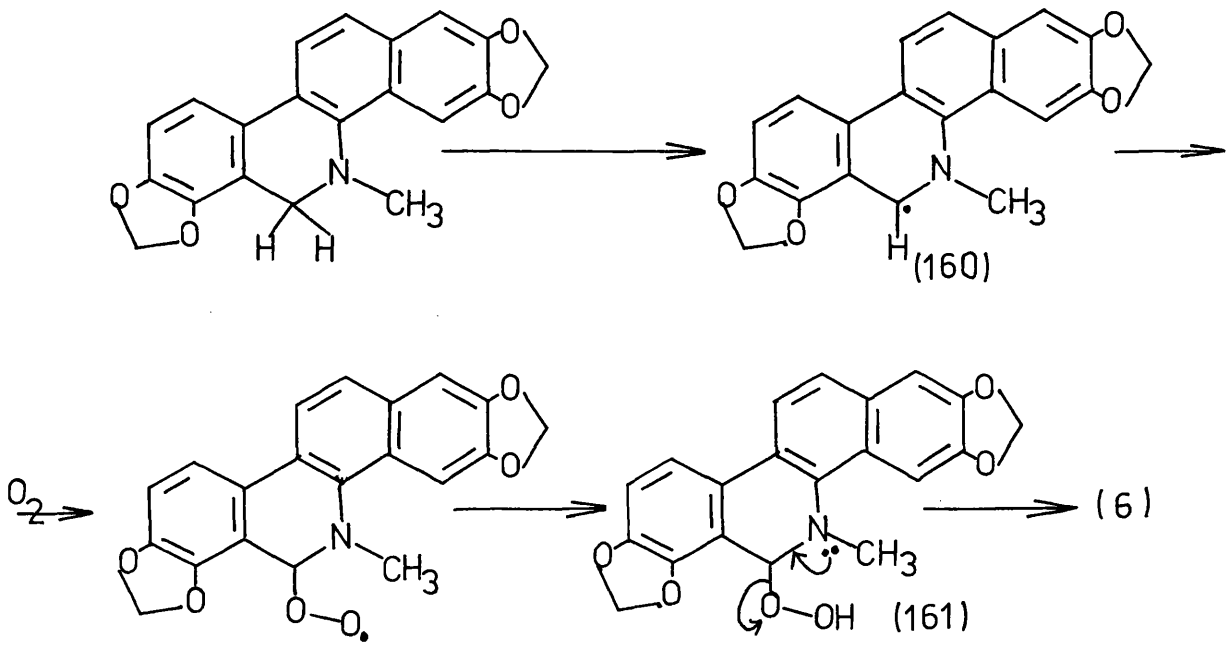
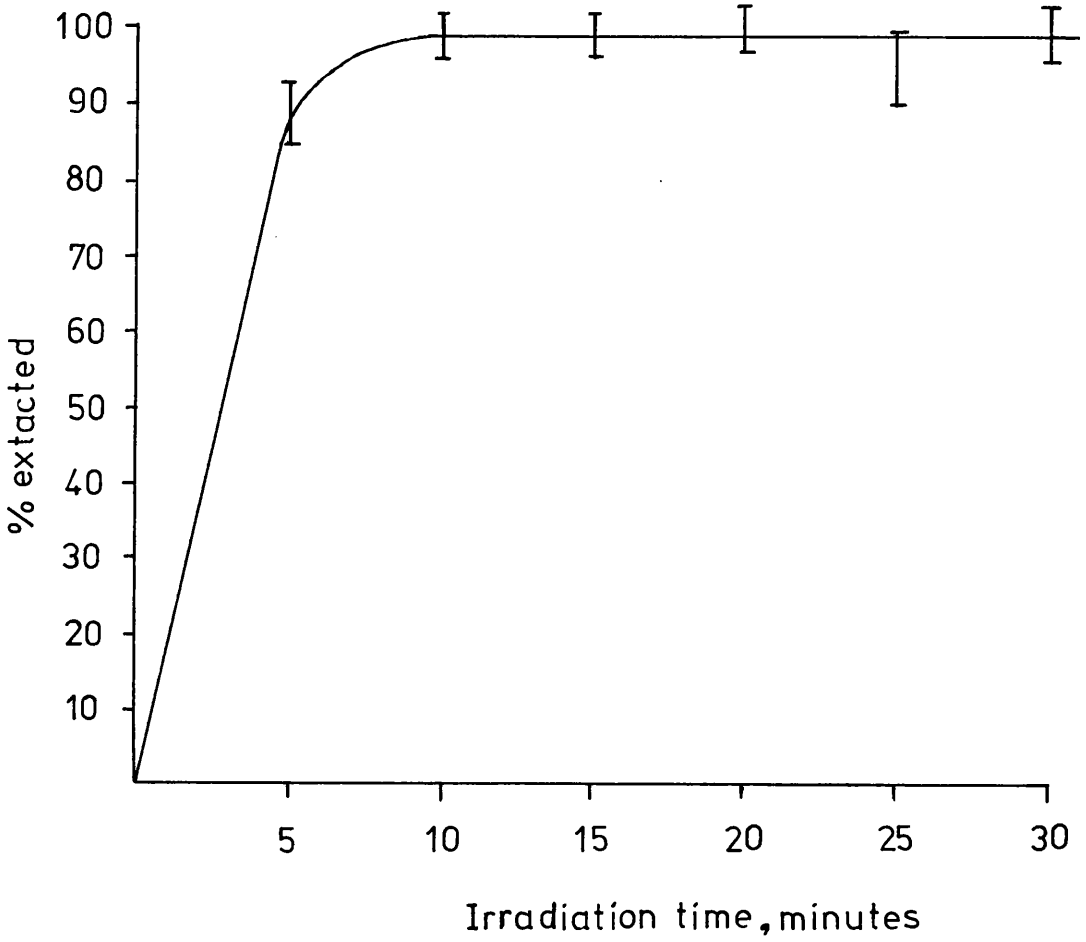


FIGURE 5

Reoxidation of dihydrosanguinarine



This was investigated by irradiation of plates for different lengths of time between 0 and 0.5 hours, samples being extracted off the plate as quickly as possible after the requisite time of irradiation. One 20 x 20cm plate containing 15 samples was examined for each time period. It can be seen from the graph, Figure 5, that the reaction is very fast, with 90% of the reoxidation being complete within five minutes.

iv) Extraction

After development of the chromatogram it was found to be necessary to include a drying stage to remove residual benzene and methanol. Three methods were investigated:

i) oven drying at 80^{0}

ii) oven drying at 50^{0}

iii) drying by cold air blast.

The results are presented in Figure 6. It can be seen that the two oven drying methods led to lower recoveries of sanguinarine than the cold air blast and the latter was adopted.

The removal of the developed sanguinarine spot was accomplished with the aid of the micro-suction apparatus shown in Figure 7: sanguinarine, absorbed onto silica gel was retained by the sintered glass disc. When all the adsorbant had been collected the apparatus was inverted, introduced into a standard 5 or 10ml volumetric flask and the sanguinarine eluted off the adsorbant, into the flask by 95% ethanol with the aid of suction.

The manipulations were investigated by delivering a known amount of pure sanguinarine onto a silica gel plate, subjecting it to the drying conditions, and extracting with the micro-suction apparatus. The u.v. absorbance of the resultant solution was compared with the u.v. absorbance of the same amount of sanguinarine delivered straight into the volumetric flask. This was repeated for a large number of replicates.

FIGURE 6

PLATE DRYING METHODS

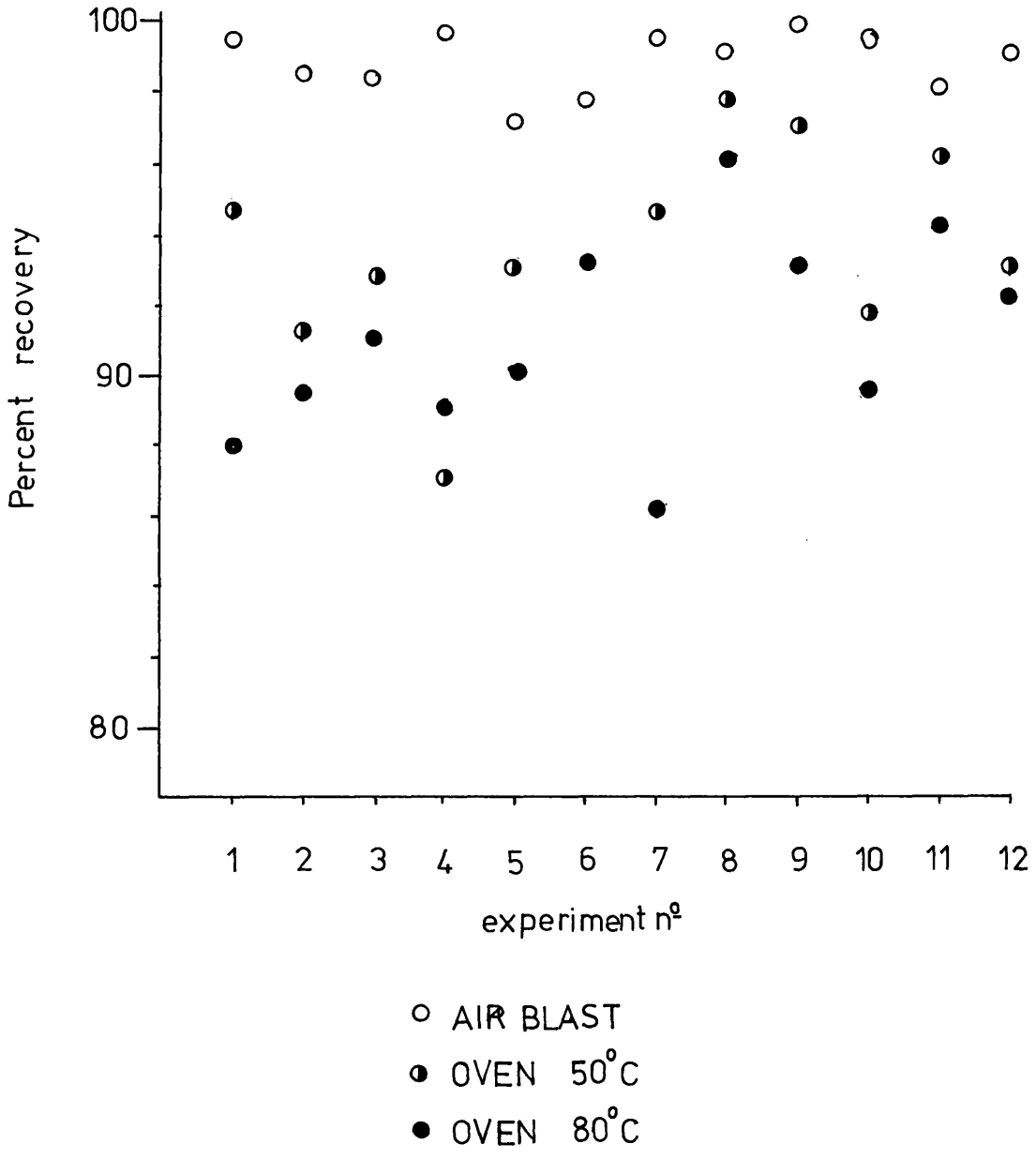
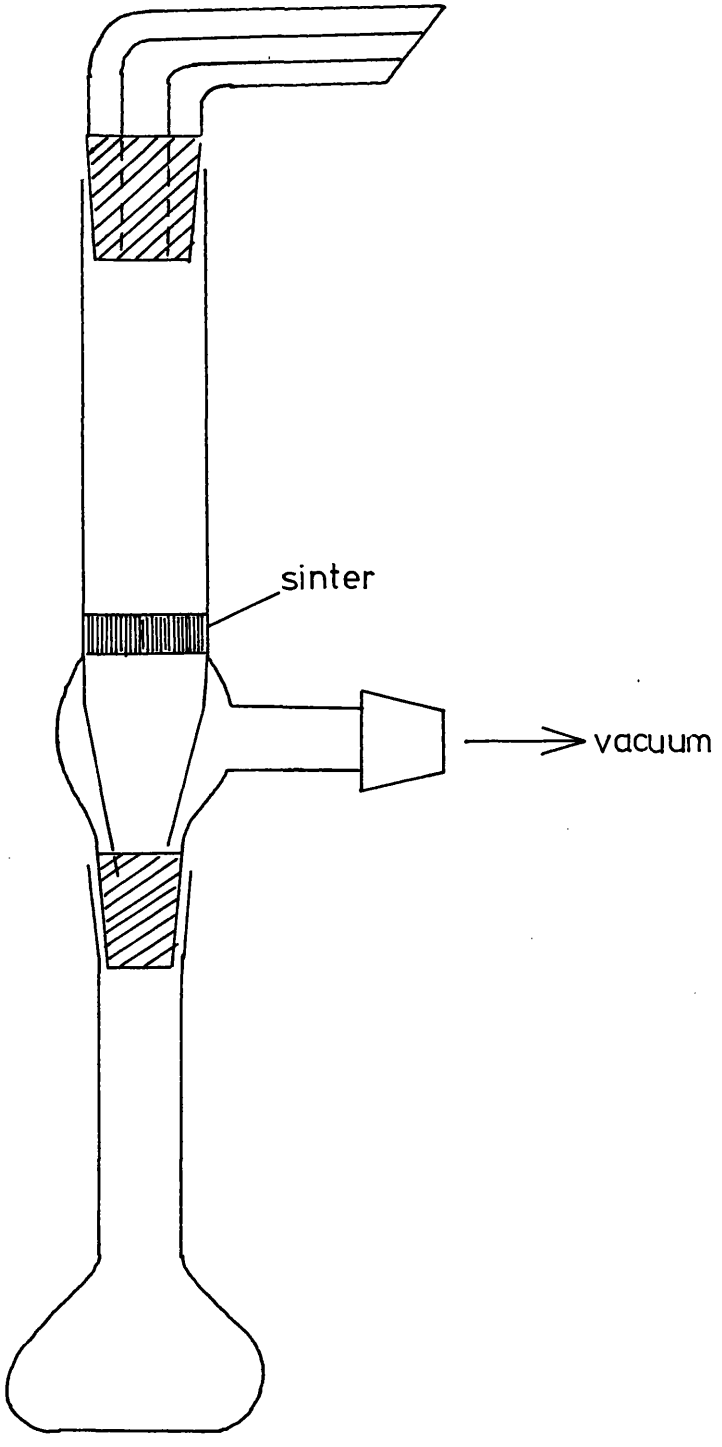


FIGURE 7

Micro suction apparatus



The results are shown in Table 12. It can be seen that the recovery off the plate has a mean value of 97.5% (range 95% - 100%).

The experiment was repeated, this time comparing the absorbance of a known amount of sanguinarine delivered directly into standard flasks with that of the corresponding amount of dihydrosanguinarine delivered into the t.l.c. plate and subjected to the oxidising conditions (15 minutes irradiation with long wavelength u.v. light) and then extracted. Results are shown in Table 13. Mean recovery is again 97.14% with a range of 95% - 101%.

A third series of experiments was carried out, comparing the absorbance of a known amount of sanguinarine with that of a corresponding amount of dihydrosanguinarine which had been spotted onto the base line of a 20 x 20cm t.l.c. plate, developed under the appropriate conditions, oxidised, dried and extracted. The results are presented in Table 14. Recovery was in the range 95% - 100% with a mean of 97%.

v) Determination of absorbance

Using pure sanguinarine chloride as a solution in 95% ethanol (acidified with one drop conc. hydrochloric acid) the adherence to the Beer-Lambert Law was investigated. A large number of replicates of different concentrations of sanguinarine were measured at a wavelength of 330nm.

TABLE 12

Recovery of sanguinarine

nominal conc^{n} µg/ml	control sample	plate sample	$\frac{\text{plate}}{\text{control}}$ %
5·0	4·90	4·65	95
10·0	9·82	9·62	98
15·0	14·80	14·80	100
20·0	20·21	19·39	96
25·0	25·30	25·03	95
22·5	22·34	21·04	99
27·5	26·95	26·95	100
		MEAN	97·57

measurement at 330 nm

TABLE 13

Recovery of sanguinarine after application as 5,6-dihydrosanguinarine

nominal conc^{n} µg/ml	control sample	plate sample	$\frac{\text{plate}}{\text{control}}$ %
5·0	5·01	4 81	96
10·0	9·87	9·55	97
15·0	14·95	14·22	95
20·0	20·05	19·84	99
22·5	22·32	22·54	101
25·0	24·80	24 04	97
27·5	27·20	25·84	95
		MEAN	97·14

measurement at 330 nm

TABLE 14

Recovery of sanguinarine after application as 5,6-dihydrosanguinarine, development and oxidation

nominal conc^{n} µg/ml	control sample	plate sample	$\frac{\text{plate}}{\text{control}}$ %
5·0	4·96	4·70	95
10·0	9·81	9·69	99
15·0	14·95	14·94	100
20·0	19·90	18·91	95
22·5	22·53	21·63	96
25·0	24·98	23·73	95
27·5	27·42	26·90	98
		MEAN	96·85

measurement at 330 nm

FIGURE 8

Absorbance vs concentration 330nm

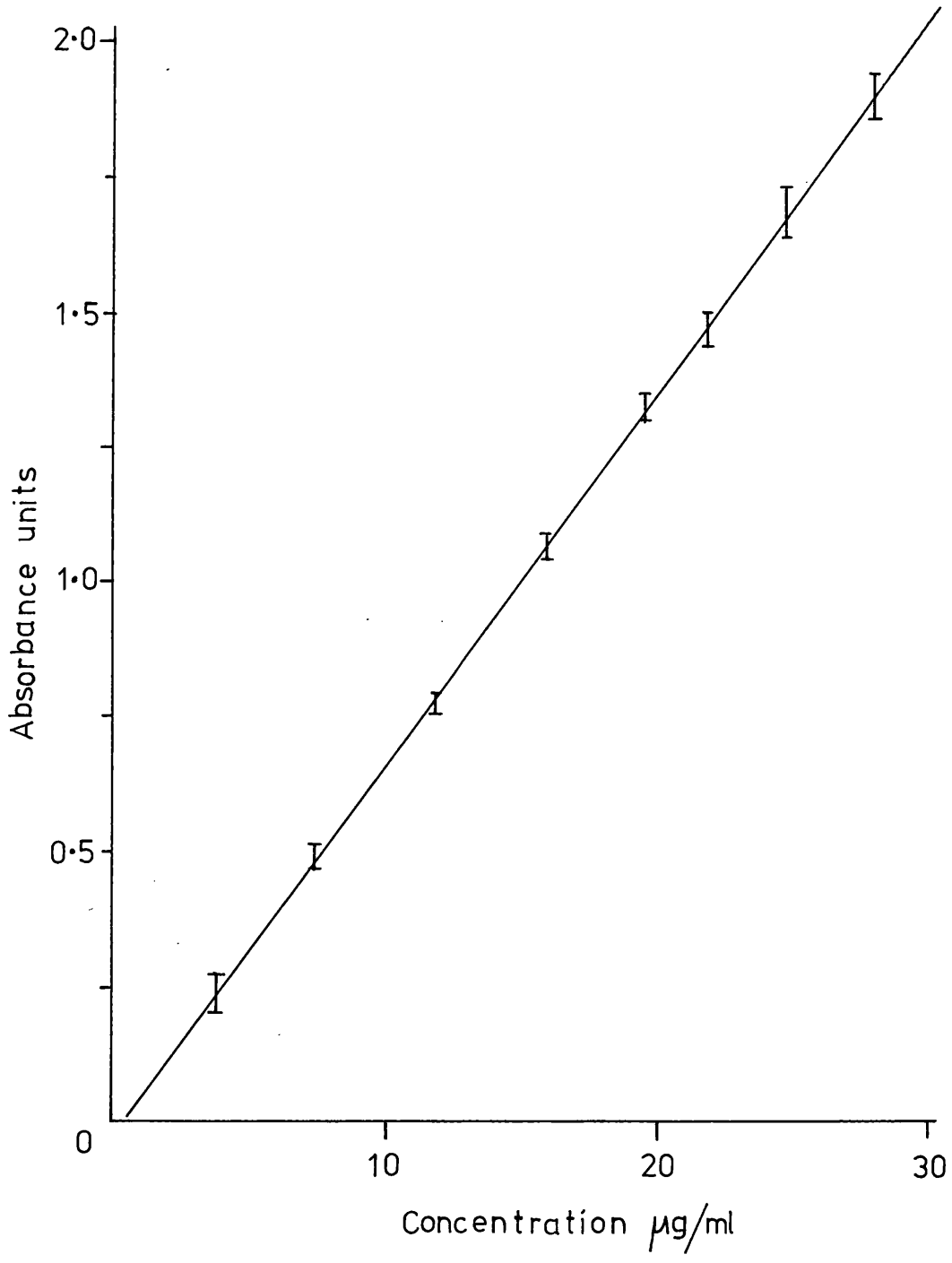


TABLE 15

Aborbance vs concentration data

concentration µg/ml	absorbance 330 nm		mean (σ)
4·00	0·22 0·20 0·27	0·25 0·23 0·25	0·237
7·50	0·51 0·47 0·50	0·48 0·47 0·49	0·487
12·00	0·78 0·75 0·75	0·79 0·75 0·76	0·763
16·10	1·04 1·08 1·07	1·05 1·07 1·04	1·058
19·60	1·34 1·30 1·33	1·30 1·31 1·33	1·318
22·00	1·46 1·48 1·46	1·46 1·44 1·45	1·458
24·80	1·65 1·74 1·68	1·62 1·70 1·71	1·683
28·00	1·90 1·92 1·93	1·86 1·91 1·87	1·898

The data obtained is summarised in Table 15, and shown graphically in Figure 8. The data was found to give a very good straight line when concentration was plotted against absorbance (correlation co-efficient $\simeq 1.000$)

Thus, sanguinarine chloride was found to obey the Beer-Lambert Law in the range 0 - 4.0mg/100ml.

vi) Efficiency of method

Several experiments were carried out to investigate the overall efficiency of the method:

a) Known solutions of sanguinarine chloride were reduced, extracted, chromatographed and eluted.

b) Suspensions of sanguinarine and of dihydrosanguinarine were subjected to the complete extraction procedure.

c) Samples of argemone mexicana seed oil and of argemone mexicana oil diluted with vegetable oil were subjected to the complete analytical method.

The results of these three sets of experiments are shown in Tables 16, 17 and 18 and in Figure 9.

It can be seen from the Tables that the method has a very high efficiency for the aqueous and ethanolic solutions of sanguinarine (97% $\pm$ 4%) and a slightly lower efficiency for the oil samples (92% $\pm$ 5%). This figure is however reproduceable.

TABLE 16

Solutions of sanguinarine chloride in ethanol

nominal conc^{n} mg/ml	applied volume µl	final volume, ml	conc^{n} mg/ml		% actual
			actual	found	
15	2	10	15·10	14·04	93
15	2	10	15·10	14·65	97
10	2	5	10·00	9·50	95
10	2	5	10·00	9·50	95
7	10	10	6·90	6·97	101
7	10	10	6·90	6·76	98
5	10	5	5·10	5·10	100
5	10	5	5·10	5·00	98

TABLE 17

Suspensions of sanguinarine chloride and dihydrosanguinarine in vegetable oil.

	nominal conc^{n} % w/w	sample weight	applied vol., µl / final vol., ml	concentration mg/ml theoretical	concentration mg/ml found	% theoretical
5,6 dihydro-sanguinarine	0·20	20	2 / 10	1·99	1·93	97
	0·02	20	10 / 5	0·199	0·187	94
sanguinarine chloride	0·50	20	2 / 50	5·00	4·55	91
	0·30	20	2 / 10	2·95	2·63	89
	0·10	20	2 / 10	0·98	0·88	90
	0·025	20	10 / 5	0·26	0·23	91
	0·01	20	10 / 5	0·10	0·09	94

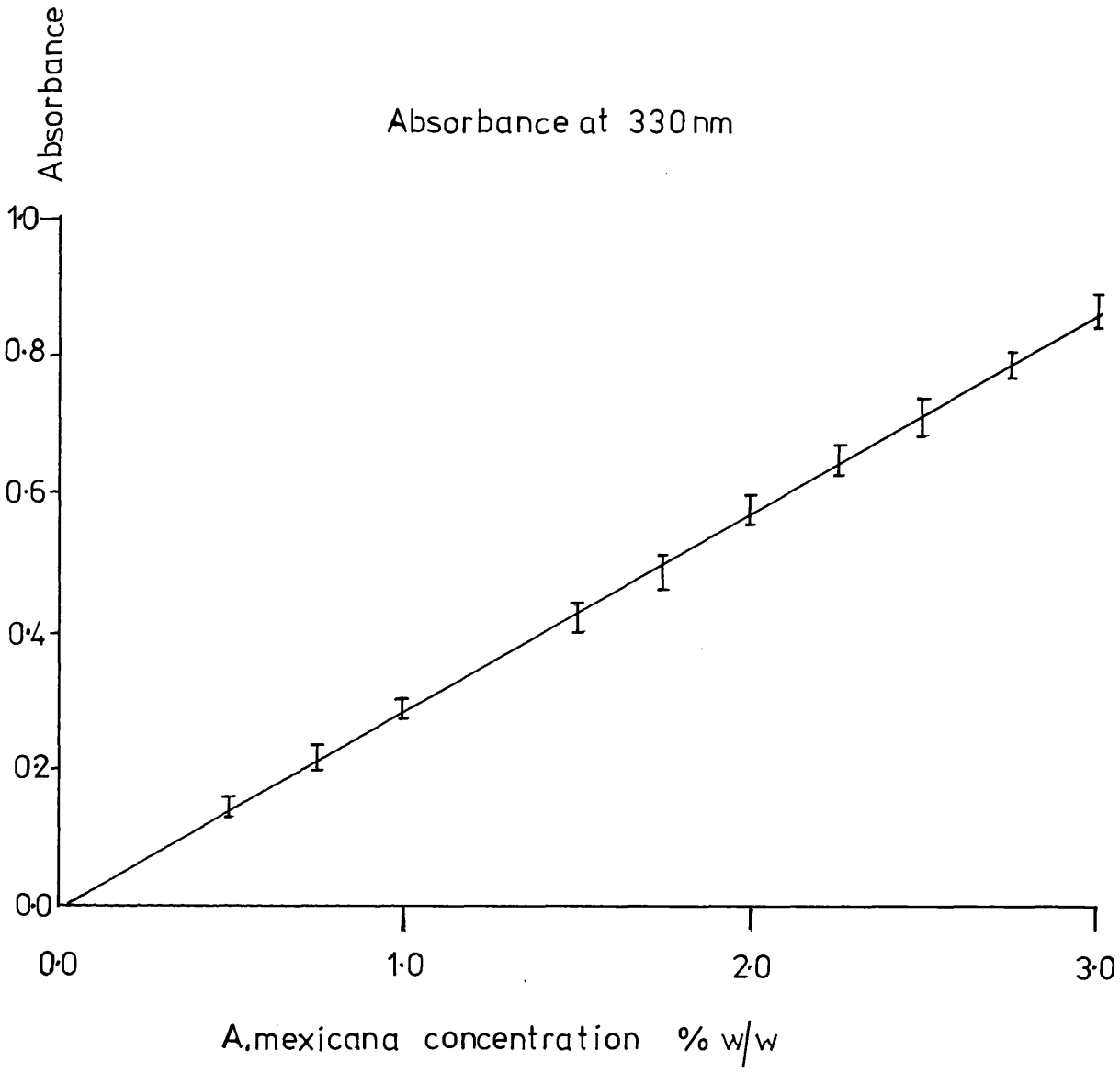
TABLE 18

Mixtures of *Argemone mexicana*[a] seed oil and domestic vegetable oil.

A.mex oil, %w/w	sample weight, gm	applied volume, µl	final volume, ml	concentration mg/gm		% theoretical
				actual	found	
3	20	10	5	0·267	0·234	88
2	20	10	5	0·178	0·164	92
1	20	10	5	0·089	0·082	92
3	10	20	5	0·267	0·259	97
0·5	30	10	5	0·044	0·041	93
0·5	20	10	5	0·044	0·040	91
0·5	20	20	5	0·044	0·041	93

a: sanguinarine content = 8·9 mg/gm determined gravimetrically

FIGURE 9


Absorbance
Absorbance at 330nm
1·0
0·8
0·6
0·4
02
0·0
0·0
1·0
2·0
3·0
A.mexicana concentration % w/w

vii) Sensitivity of Test

a. Non-quantitative

Sanguinarine exhibits an intense orange fluorescence under long wavelength ultra-violet light; using a combination of this and the R_f value to identify sanguinarine it is possible, by a series of dilutions to identify the alkaloid at a concentration of 1ppm.

b. Quantitative

The lower useful limit is obviously dependent on the amount of sample available since this controls the amount of sanguinarine available for extraction, etc. The limit is set by the fact that the chromatograph sample must contain 10^{-5}gm dihydrosanguinarine.

RAPID SCREENING PROCEDURE

A simple screening procedure was developed for rapid examination of the gross sample. It was designed mainly for screening oil samples but could be used with other fluids. The method uses the principle of two-dimensional chromatography on thick layers of silica gel. A sample of the oil is applied to one corner of a 20cm x 20cm thick (750μ) plate and developed in benzene over 10cm. The plate is then removed, dried in a cold air blast, rotated through 90^{o} and developed in a benzene/methanol (6:1) solvent mixture. If present, sanguinarine appears as an orange spot of R_f co-ordinates (0.00, 0.70-0.80).

Samples giving a positive result would then be submitted

to the quantitative determination described above.

Examination of physiological material from cases of known Argemone mexicana poisoning

Samples of blood and urine were provided by Dr Hakim from one of his patients in a Bombay hospital. The patient, a young man named Haresh Adhyra, had been the victim of acute Argemone mexicana poisoning[151]. By examination of the material it was hoped to determine the persistence of sanguinarine after removal of the source.

Unfortunately, it was found that the samples were two years old when received and had been subjected to varying amounts of pretreatment. The material comprised blood and urine samples obtained over a period of two months, between August and October 1973.

Sanguinarine was found in low concentrations in the blood sample for August, but not subsequently. No sanguinarine was detected in the urine.

This finding may have some significance since it indicates that sanguinarine can remain in the blood-stream for between four and eight weeks, and that it is not readily metabolised or excreted. This lends some support to the theory of Hakim that

sanguinarine is "trapped" in the system and circulated, being absorbed through the gut, circulated through the blood and then secreted in the oesophagus. Sanguinarine has been implicated in the genesis of cancers of the stomach and oesophagus (q.v.). Results are shown in Table 19.

TABLE 19

HUMAN PATHOLOGY

Accredited case of A.mexicana poisoning

	sample	date	weight gm	sanguin-arine[a]	benz(c)-acridine[b]	others[c]
BLOOD	1	17.7.73.	30	absent	absent	absent
	2	17. 8.73	90	present	"	"
	3	undated	60	absent	"	"
	4	9.73	20	"	"	"
	5	10.73	25	"	"	"
URINE	6	7.73	86	"	"	"
	7	8.73	80	"	"	"
	8	10.73	14	"	"	"
	9	undated	20	"	"	"

a: tlc method

b: tlc and glc

c: tlc

MINOR ALKALOIDS OF SEED OIL OF ARGEMONE MEXICANA, LINN.

Seed oil (200g) was extracted with 95% ethanol (800ml) for several days, after which it was separated from the oil and the ethanol removed under reduced pressure. The residue, after dissolving in chloroform (200ml) was extracted with 2M hydrochloric acid (5 x 100ml). The combined acid portions were basified with sodium bicarbonate solution and extracted with chloroform (5 x 100ml). After washing with water (2 x 100ml) and drying over magnesium sulphate the chloroform was removed under reduced pressure to leave a basic residue (600mg).

The residue was chromatographed on thick layers of silica gel (1mm) using benzene/methanol (6:1) as developing solvent. The upper 80% of the orange sanguinarine band was removed and discarded; the remainder of the adsorbant was removed and extracted with 95% ethanol. After evaporation of the ethanol under reduced pressure the residue was subjected to tlc analysis using the following solvent systems (silica gel adsorbant):

A benzene/methanol, 6:1

B benzene/methanol, 3:2

C benzene/acetone/methanol, 7:2:1

Authentic samples were used as references. The alkaloids were visualised with ultra-violet light, removed from the plate

using the micro 'vacuum cleaner' and eluted with 95% ethanol. Ultra-violet spectra were recorded.

ALKALOID	(R_f: A,B,C)	λ_{max} nm	COLOUR
chelerythrine	(0.76,0.72,0.67)	228,280,319sh.	gold
berberine	(0.00,0.00,0.00)	230,265,345	green
protopine	(0.35,0.27,0.25)	240sh,290	white

identical with authentic samples.

QUANTITATIVE DETERMINATION OF SANGUINARINE

The sample of oil (A. mexicana seed oil or cooking oil) (5-30 g, as appropriate) was dissolved in petroleum ether (b.p. 40-60°) and a solution of sodium borohydride (500 mg) in ethanol (10 ml) was added with stirring. After stirring for 15 min the turbid liquid was treated with 2 M hydrochloric acid. The resultant lower acid layer was collected and the upper organic phase was extracted twice with 2 M HCl. The combined acid layers were basified ($NaHCO_3$ solution) and extracted into chloroform. The dried chloroform extract was evaporated, the residue was dissolved in ethanol, and the volume was adjusted to 1.00 ml in a standard flask.

Samples (2-20 µl) of this ethanol solution were spotted onto a 20 x 20 cm plate coated with silica gel (250 µm) in the usual way.

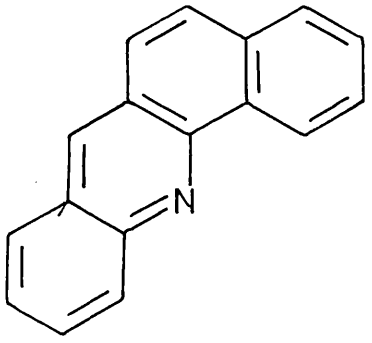
The spot runs were then scored and the chromatogram was developed over 10 cm with benzene-methanol (6:1). The plate was dried (air blast for 5 min), then irradiated with long-wavelength UV light for 15-20 min to effect oxidation of dihydrosanguinarine to sanguinarine. The latter appeared as an orange spot at R_f 0.78. This was removed from the plate with the micro suction apparatus, dissolved in ethanol containing one drop conc. HCl and the solution made up to 5.00 ml in a volumetric flask. The absorbance of this solution at 330 nm was measured and the concentration of sanguinarine computed from a calibration curve of concentration of pure sanguinarine chloride vs. extinction at 330 nm. Usually ten samples of the test solution were processed on one plate and the average concentration was calculated.

CHAPTER 3

THE ROLE OF SANGUINARINE IN THE PRODUCTION OF CANCER OF THE STOMACH AND OESOPHAGUS

Introduction

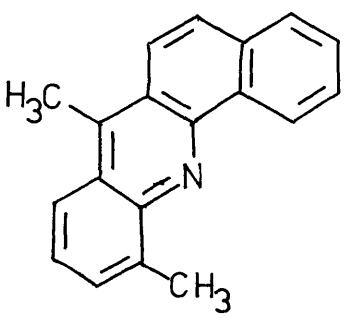
In a series of experiments designed to investigate the transmission of orally administered sanguinarine to milk and urine, Hakim et al[114] noted the presence of several compounds which were absent from the milk and urine of control subjects. One of these compounds was investigated and identified, purely on the basis of its ultra-violet spectrum and electrophoretic behaviour, as being benz(c)acridine (161)



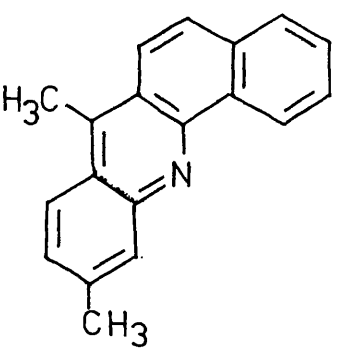
(161)

Further experiments showed that the metabolite was present in greatest quantities in the livers of animals fed on sanguinarine rich diets. Perfusion of an isolated rat's liver with blood to which sanguinarine in saline had been added led to the release of the 'metabolic' product in four hours. Hakim states that this product must be derived from sanguinarine since it was always absent in control studies.

Having 'identified' the metabolite as benz(c)acridine Hakim and his co-workers realised the carcinogenic implications of a compound in this class of polynuclear heterocyclic hydrocarbons which had already been synthesised by Lacassagne.[152] Some members of this group, for example, 6,10-dimethylbenz(c)acridine (162) and 7,10-dimethylbenz(c)acridine (163) showed intense carcinogenicity.[152,153]



(162)



(163)

Experiments were undertaken by Hakim[154] to induce cancers in experimental animals both by feeding of Argemone oil and sanguinarine, direct i.v. injection of oils, and by implanting benz(c)acridine in wax pellets into the bladders of rats. Increases in the numbers of cancers over the controls were claimed.

Hakim[154] implicates benz(c)acridine as the cause of oesophageal and of stomach cancers on the basis that sanguinarine is actively secreted into these organs after systemic absorption, although no evidence is offered for the

similar secretion or concentration of benz(c)acridine in either organ.

Finally, Hakim[108,154] presents demographic data to correlate the incidence of gastric cancers with climate, geographical location and social class or race. He shows, for instance, a variation in the incidence of oesophageal cancer with edible oil usage of different Indian social classes[155] (linking oesophageal cancer with the adulteration of edible oils described above). Quoting the study of Kmet and Mahboubi[156] on the distribution of oesophageal cancers in the coastal areas of the Caspian Sea in Iran, Hakim notes that the incidence of this cancer is relatively high in the drier regions as compared with the wet, tropical regions, and points out[157,158] that this distribution is similar to that of the Iranian papaveraceae.

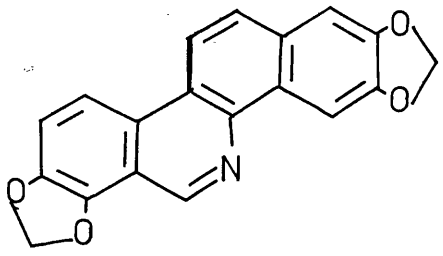
A considerable amount of evidence of this nature is proposed but it must be regarded with suspician because of the variety of sources and the lack of any scientific control over the data gathering: sudden increases in the incidence of a particular disease may simply indicate the upgrading of local health care or a renewed medical interest in specific diseases. Similarly, differences of disease incidence between social classes or races may indicate differences in the standard of health care available or in the attitude of a particular race or class to health care.

The search for environmental causes for diseases must be investigated in a controlled, scientific manner over many years, and not on the basis of fortuitous coincidences as appears to be the case with benz(c)acridine.

In order for benz(c)acridine to be produced from sanguinarine *in-vivo* the following transformations must take place, although not necessarily in the order shown below:

1. N-demethylation
2. a) cleavage of the methylenedioxy groups followed by
   b) dehydroxylation
3. rearrangement of the benzo(c)phenanthridine skeleton to that of benz(c)acridine.

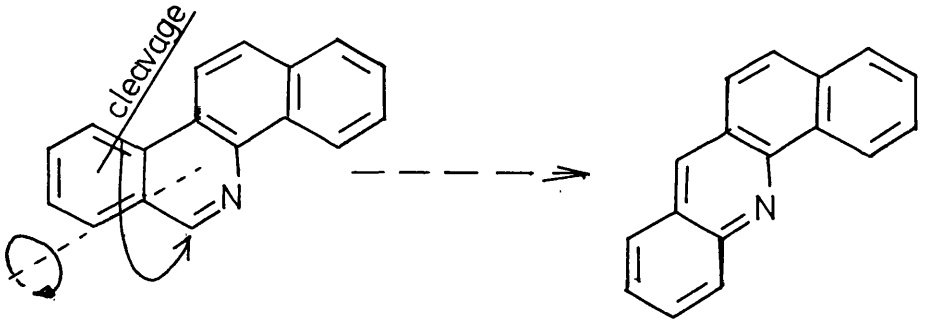
Certainly, the reactions 1, 2.a) and 2.b) are known [159] *in-vivo* transformations and the N-demethylation product of sanguinarine, norsanguinarine (10), has been observed in extractsof monkey urine.



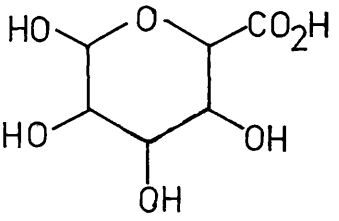
(10)

Products derived from reactions 2.a) and 2.b) were not identified, although they should have been present if this type of detoxification system is operating.

The rearrangement of the benzo(c)phenanthridine is a profound metabolic transformation of a type which has not been reported <u>in-vivo</u>, although there are some <u>in-vitro</u> analogues.[160-162] The transformation would be most simply achieved by re-orientation of the benzo(c)phenanthridine A-ring as shown below:



It must be asked why this type of reaction should occur and it is difficult to put forward any reason unless it is that the shape of the sanguinarine molecule closely mimics some other molecule of physiological origin which undergoes this reaction. Under normal circumstances metabolism takes place in the endoplasmic reticulum of the liver and is essentially a hydroxylation process; introduction of a hydroxy group is usually followed by conjugation, for example, with glucuronic acid (164).



164

Since sanguinarine readily forms its 6-hydroxydihydro- derivative, this could form the glucuronide and the sanguinarine excreted essentially unchanged. Alternatively, the 6-hydroxy- derivative could be removed by conjugation with a thiol residue of a protein.[164]

Since it is possible for the body to deal with sanguinarine simply it is unlikely to be involved in major structural changes. Another possibility exists, which is that sanguinarine triggers the release of other compounds or stimulates their production by interfering with biochemical processes. An example is provided by the production of neurohormones in the eye after stimulation of the hypothalamus by sanguinarine, mentioned above (p.92).

A final possibility is, of course, that benz(c)acridine is not present and that its earlier identification by Hakim et al.

was a case of mistaken identity. In this context it should be remembered that the only criteria of identity were the ultra--violet spectrum, the electrophoretic behaviour, and the comparison of these properties with an authentic sample. Although this is sufficient in less critical applications, it is simply not enough evidence on which to base a theory with such far--reaching implications.

It is quite possible that in experiments in which the ingested substance was Argemone oil or foliage, the 'metabolites' could simply be trace alkaloids of Argemone mexicana. This could also be the case when sanguinarine chloride was used since the source of the material used in Hakim's work was invariably extracts of A. mexicana.[151]

In studies carried out by the author, benz(c)acridine has not been found on any occasion in any samples of blood or urine which were obtained during the course of feeding experiments with cats, rabbits or monkeys using either A.mexicana seed oil or sanguinarine (samples provided by Dr Hakim). Benz(c)acridine was not found in the organs or viscera of animals sacrificed after feeding experiments. The same result was obtained both for chronic and for acute studies.

Similarly, benz(c)acridine was not found in samples of sheep livers obtained by Dr Hakim from Iceland, an area where

the incidence of gastric cancers is high and where the available grazing land had a high density of papaveraceous plants.

It is considered by this author that the 'metabolites' detected by Hakim et al are not products of metabolism but extraneous alkaloidal material which has been concentrated by the liver. This is not improbable, since no attempts at purification of the sanguinarine chloride samples were made.[151] In all cases where extracted alkaloid was used a mixture mainly of sanguinarine chloride and dihydrosanguinarine hydrochloride was obtained by treatment of a dry ethereal solution of seed oil with dry hydrogen chloride gas. The whole of the precipitated material was oxidised by aqueous ferric chloride and the quarternary chloride recrystallised from 2M hydrochloric acid. It has been found that sanguinarine chloride obtained by this method contains several impurities among them berberine, protopine and chelerythrine. The identification data quoted by Hakim et al, which is an ultra-violet spectrum and some electro-phoresis data could equally be applied to berberine, which also fluoresces green under uv light.

Initial work concentrated on developing a method for the rapid determination of benz(c)acridine. It was soon discovered that benz(c)acridine was very amenable to glc analysis, rapidly eluting from an SE30 (silicone) column at relatively low (215^{o}C) temperature. It was also found that benz(c)acridine

could be separated from a crude mixture of monkey urine bases obtained from urine concentrates by direct chromatography of a chloroform solution of the crude bases. (Figures 10 and 11)

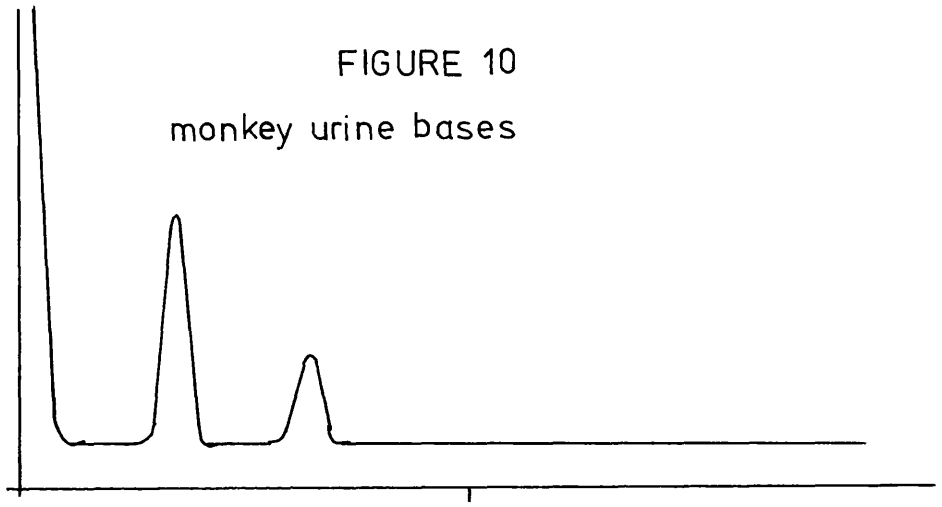


FIGURE 10

monkey urine bases

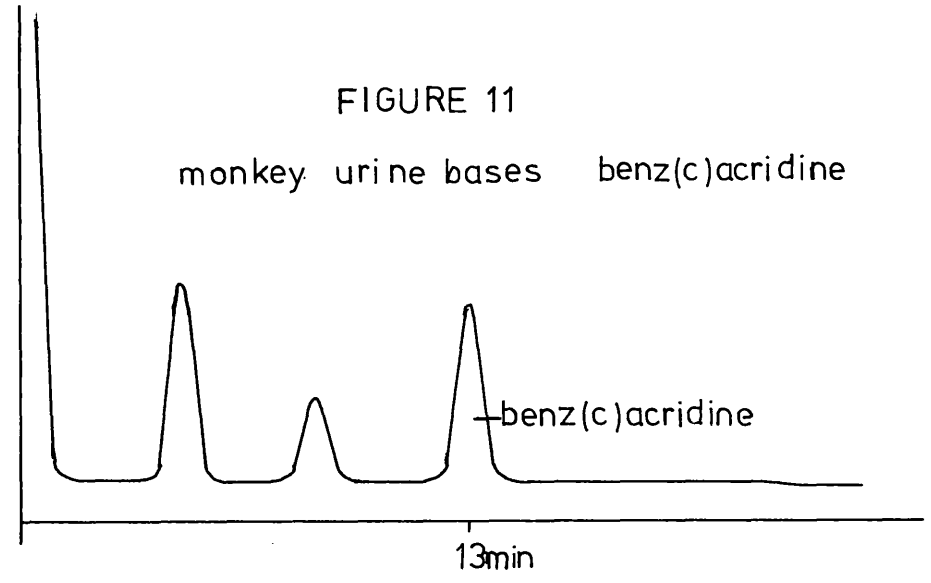
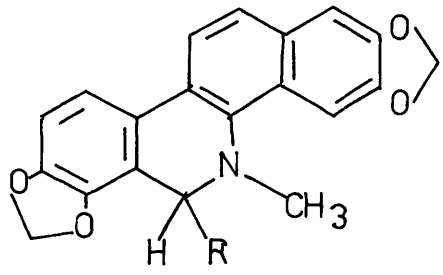


FIGURE 11

monkey urine bases benz(c)acridine

Since the method was so simple for benz(c)acridine it was hoped that it could be extended to include sanguinarine. It was realised that the ionic nature of the aromatic compound would preclude gas chromatography and that the only possibility was to form a derivative more amenable to this type of analysis. Since attack at the 6-position to give 5,6-dihydro derivatives

is favoured, several of these were prepared for chromatographic study.



R=OH
H
CN
CH_2COCH_3
CH_3
β-phenylhydrazine

(165)

The pseudobase (165 R=OH) was found to be thermally unstable, as was the pseudocyanide (265 R=CN). Silyl derivatives of the pseudobase could not be prepared. The 6-methyl- and 6-β-phenylhydrazinyl- derivatives were made but did not produce suitable chromatograms. Initial results with the 6-acetonyl-derivative (165 R=CH_2COCH_3) were encouraging but it was soon realised that the chromatograms obtained were due to thermal decomposition products. 5,6-dihydrosanguinarine (165 R=H) was found to elute, but as a very broad peak at elevated temperatures and extended times, thus making it unsuitable for analysis.

The search for a glc method for sanguinarine was abandoned since the tlc approach was by this time giving extremely good results. It was, however, retained for benz(c)acridine screening.

EXAMINATION OF PHYSIOLOGICAL SAMPLES

Monkey urine collected during several feeding experiments using A.mexicana oil was presented for examination as dried residues. After rehydration with water the samples were subjected to acid hydrolysis to break down any protein complexes; neutralisation was followed by treatment with sodium borohydride which, after fifteen minutes, was decomposed by the addition of dilute acid. The solutions were worked-up for bases in the usual way. The crude residue of monkey urine bases was made up to standard volume with chloroform and subjected to analysis for sanguinarine by tlc analysis (see Chapter 2) and for benz(c)acridine by glc analysis.

Results from Monkey 121 showed that sanguinarine is excreted in the urine of animals fed on sanguinarine rich diets, although not in the quantities that the feeding rate would lead one to expect. This attenuation of sanguinarine concentration can be ascribed to several mechanisms:

i) non-absorption from the gut: majority of sanguinarine excreted in faeces

ii) removal of sanguinarine as protein complex in serum and thus reduced availability for excretion

iii) metabolism

iv) concentration in an organ or organs.

TABLE 20

EXAMINATION OF TISSUE SAMPLES

MONKEY NO. 122

Organ	sanguinarine	benzacridine	others	identity
lung	x	-		
liver	x	-		
kidney	x	-	x[a]	mixed Argemone alkaloids
testes	-	-		
heart	-	-		
spleen	-	-		
muscle	-	-		

a = green fluorescent tlc spots

MONKEY Nº 121

organ	sanguinarine	benzacridine	others	identity
liver	x	—	—	
urine	x	—	x	as above

x present

– absent

An investigation was undertaken into the possibility of concentration in organs (iv) and the organs of a monkey (Monkey No. 122) which had been fed on a sanguinarine rich diet for thirty days prior to death were examined. The organs, which were presented in dehydrated form, were ground in a laboratory mill prior to rehydration and acid hydrolysis. The standard analytical procedure was followed. Although sanguinarine was found in the lungs, liver and kidney, it was not found in sufficient quantities to explain the attenuation of sanguinarine. Surprisingly, none was found in the blood. The testes, heart, spleen and some muscle tissue was also examined but no interesting compounds were found.

Identical spots on the tlc chromatograms of the kidney extracts of Monkey No. 122 and in the liver and urine of Monkey No. 121 were found to exhibit green fluorescence under ultra-violet light and attempts were made to isolate these by preparative thin layer chromatography. Work-up of the relevant area of the preparative plates produced very small amounts of material, which on subsequent tlc exhibited total loss of the original component: mixtures were obtained in which Argemone components were obvious. Results are summarised in Table 20.

The major factor in the attenuation of sanguinarine would be expected to be nonabsorption (i) since the sanguinarine was presented to the system as a solution in oil; its bioavailability would be relatively low and would be very dependent on

the residence time in the stomach as it is only there that the pH is sufficiently low to extract the alkaloid from the oil phase. Collection and analysis of urine and faeces would have allowed the degree of absorption to be assessed but samples were not made available.

Serum protein binding is accepted as a major contributor to the attenuation of pharmacologically active substances after absorption into the blood. The extent of serum protein binding was impossible to assess: since the degree of absorption was not known it was not possible to assess the difference between the amount absorbed and the amount excreted.

Problems were encountered in analysing the urine extracts for metabolites since the feeding regimes had been carried out using either the expressed seed oil of Argemone mexicana or a crude mixture of alkaloid chlorides and hydrochlorides precipitated from an ether solution of the seed oil. Although sanguinarine was the major component in both cases the presence of extraneous alkaloidal material, also capable of systemic absorption and subsequent excretion, complicated matters considerably, in that it was difficult to establish the source of any material other than sanguinarine extracted from the urine. The appearance of a tlc spot could simply signify the preferential excretion of a minor component of the original feedstuff rather than the metabolism of the sanguinarine. The

absence of material from control animals also hampered the interpretation.

Examination of liver tissue

Liver samples were provided in dehydrated form and were ground in a laboratory mill prior to rehydration and acid hydrolysis. The standard analytical procedure was followed and samples were subjected to tlc analysis for sanguinarine, and glc analysis for benz(c)acridine. The results are shown in Table 21. It can be seen that sanguinarine was tentatively identified in three out of the forty-one samples. Benz(c)-acridine was not found in any of the samples, but an unidentified compound was detected in two cases. Small sample size precluded any confirmatory studies.

It may be significant that sanguinarine was detected in three of the samples since Iceland is a major supplier of liver and liver products to Europe. However, key information concerning the origin and history of the samples was held by Dr Hakim and was not made available before his unfortunate death. The Sola Hakim Centre for Medical Research where records were presumably kept has also ceased to function.

RESULTS OF INVESTIGATIONS OF LIVER SAMPLES:

ICELANDIC SHEEP

SAMPLE	WEIGHT(GM)	RESIDUE WT.(MG)	SANGUINARINE	BENZACRIDINE
1	16.5	7	absent	absent
2	14.8	1	x	"
3	14.6	2	absent	"
4	14.7	2	x	"
5	15.8	6	absent	"
6	14.2	1	x	"
7	14.3	8	b	"
8	15.2	4	absent	"
9	13.2	5	"	"
10	15.5	5	"	"
11	14.1	4	"	"
12	13.8	4	"	"
13	14.7	6	"	"
14	14.9	7	"	"
15	14.5	5	"	"
16	16.1	3	"	"
17	14.6	5	b	"
18	14.5	12	absent	"
19	14.0	6	"	"
20	15.1	3	"	"
21	12.5	7	"	"
22	11.6	3	"	"
23	15.7	6	"	"
24	12.2	8	"	"
25	11.4	4	"	"
26	13.5	3	"	"
27	11.2	2	"	"
28	11.0	5	"	"
29	11.9	8	"	"
30	12.6	4	"	"
31	12.6	4	"	"
32	9.7	5	"	"
33	9.2	3	"	"
34	8.2	1	"	"
35	9.0	4	"	"
36	8.0	2	"	"
37	9.0	1	"	"
38	8.2	3	"	"
39	8.5	3	"	"
40	9.3	3	"	"
41	8.7	5	"	"

b = unidentified gold spot R_f 0.55

EXPERIMENTAL

GAS-LIQUID CHROMATOGRAPHY OF BENZ(C)ACRIDINE

Using material prepared as above and recrystallised three times from ethanol, solutions were made up, in chloroform, of the following concentrations:

.5mg/litre

5mg/litre

50mg/litre

500mg/litre

5000mg/litre

2 µl aliquots were applied to a glc apparatus and the peak areas calculated. 10 replicates of each concentration were chromatographed. Mean peak area was plotted against concentration and a straight line fitted. The calibration curve obtained is shown in Figure 12.

Benz(c)acridine was added to solutions of extracts of dried urine and liver prior to chromatography. The expected amount of benz(c)acridine was detected in the chromatogram.

Benz(c)acridine wasadded to portions of reconstituted

dried urine prior to extraction. For the purposes of this study, reconstituted urine was extracted both by

a) a standard extraction procedure for basic material

b) the sodium borohydride treatment described above.

In both cases the extraction rate was nearly 98%. No statistically significant difference was found between the two procedures. Results are summarised in Table 22.

TABLE 12

Gas chromatography of benz(c)acridine

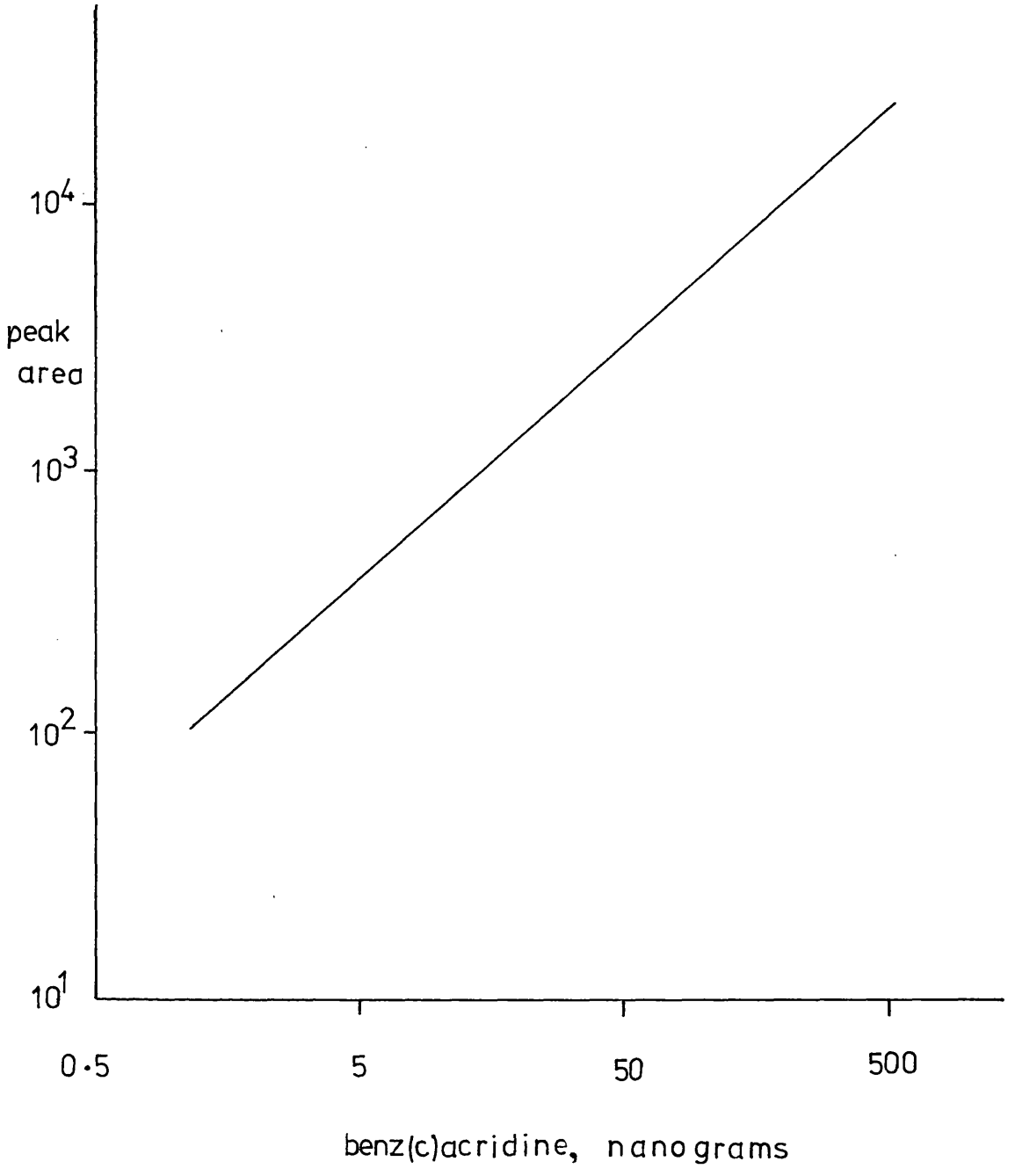


TABLE 22

Extraction of benz(c)acridine: recovery of material added to reconstituted dried monkey urine

i standard base work-up

ii sodium borohydride procedure

amount added[a] to 1·0 gm dried monkey urine	i) standard		ii) borohydride	
	mg/gm	%	mg/gm	%
1·0 mg	·984	98·4	·980	98·0
0·5 "	·494	98·7	·495	99·0
0·3 "	·296	98·7	·295	98·5
0·1 "	·099	99·1	·098	98·4
0·05 "	·0495	98·9	·0491	98·2
0·03 "	·0291	97·1	·0292	97·4
0·01 "	·0096	96·3	·0097	96·9

a: solution in $CHCl_3$

Preparation of benz(c)acridine (161)

1-naphthol (12.0gm,0.083M) and ortho-toluidine hydrochloride (11gm,0.076M) were intimately ground together and then heated at 230°C for 4.0 hours. After cooling the mass was treated with warm 20% potassium hydroxide solution (200ml) and toluene (200ml) and allowed to stand for twenty four hours. The layers were separated and the aqueous layer extracted with toluene (3 x 200ml). The combined toluene extracts were washed with water (2 x 200ml), dried over magnesium sulphate and the toluene removed under reduced pressure to leave an oil (8.0 gm). Vacuum distillation at 0.3mm.Hg, 160°C produced an oil, (6.25gm).

The product of the above reaction (6.0gms) and lead oxide (60gm,0.27M) were heated at 240°C for four hours. After cooling the mass was extracted with diethyl ether (200ml). Removal of diethyl ether under reduced pressure left a brown gum (3.0gm) which was treated with hot 2M hydrochloric acid and stirred for 0.5 hours after which time the hot solution was decanted. On cooling a yellow crystalline precipitate was formed. Treatment with 2M sodium hydroxide solution to alkaline pH was followed by extraction with chloroform (3 x 100ml). The combined organic extracts were washed with water (2 x 100ml) and dried over magnesium sulphate. Removal of chloroform under reduced pressure produced a solid which was recrystallised from ethanol to give benz(c)acridine as yellow crystals (2gms, 10% on 1-naphthol). MPt 106-108°C ν_{max} 1570cm^{-1}, 1590cm^{-1} M^+ 229.

CHAPTER 4

INVESTIGATIONS INTO THE SYNTHESIS OF RADIOLABELLED SANGUINARINE DERIVATIVES

In order to investigate the proposed in-vivo rearrangement of sanguinarine to benz(c)acridine it was decided to synthesise derivatives of sanguinarine containing a ^{14}C label in the benzo(c)phenanthridine skeleton. The label would have two functions:

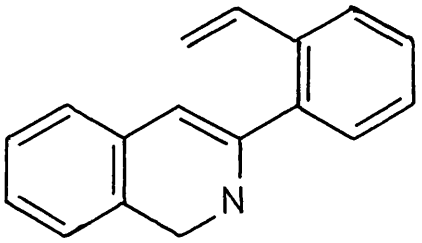
i) it would confirm that any metabolite found was derived from sanguinarine

ii) it would throw some light on the mechanism of the proposed rearrangement reaction since the position of the label in the metabolite could be determined by degradation.

Since the ^{14}C label was to act as a metabolic tracer it was important that it should not be in an easily metabolised, reactive position. This precluded the substitution of the sanguinarine 6-position, the N-methyl group or either of the methylenedioxy carbon atoms. Similarly, the ^{14}C label might have been lost had it been placed in either of the peripheral benzene rings. It was decided that the most suitable position for both avoidance of label-loss and simplicity of synthesis would be either the C-11 or C-12 positions.

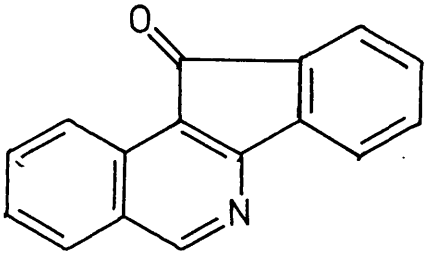
Two synthetic approaches were considered. The first involved the synthesis of the vinyl compound (166) derived from a protopine type of compound. Removal of the terminal

methylene group and its replacement by a ^{14}C labelled group would be followed by photocyclisation after the method of Onda.[5]



(166)

The second method involved ring expansion of the indenoisoquinoline (167) with radiolabelled diazomethane.



(167)

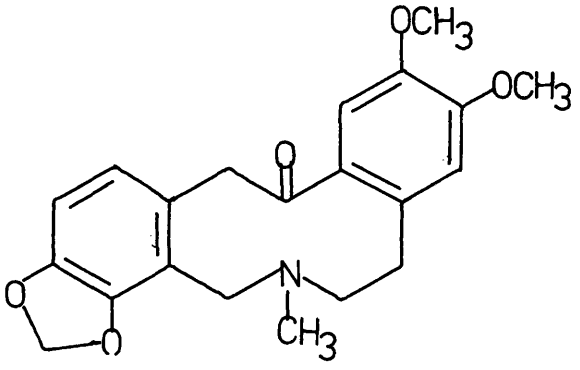
The initial work was intended to establish the synthetic routes using non-radiolabelled reagents. Part I describes the attempts to modify the vinyl group of (166), Part II describes the synthesis and attempts at ring expansion of (167).

PART I

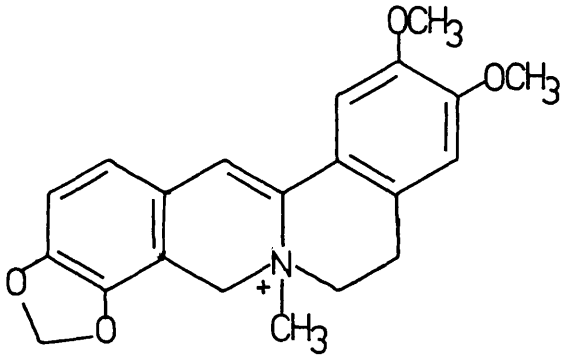
Due to its availability, cryptopine (168) was used as a model compound in this study.

The conversion of cryptopine to the vinyl compound, anhydrocryptopine (170) via the protoberberine (169) is a simple,

known reaction.[166,167]



(168)



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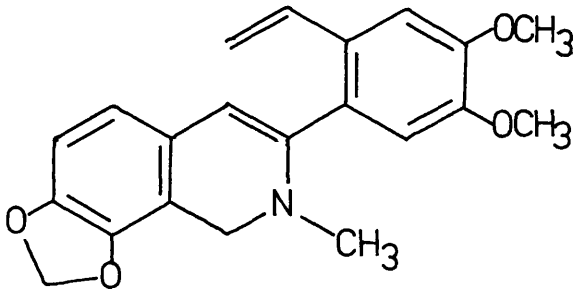


(170)

Cyclisation of (168) with $POCl_3$ is followed by Hofmann degradation with methanolic potassium hydroxide to give the vinyl compound, anhydrocryptopine. Irradiation with ultra-violet light followed by catalytic dehydrogenation would give the fully aromatic benzo(c)phenanthridine.

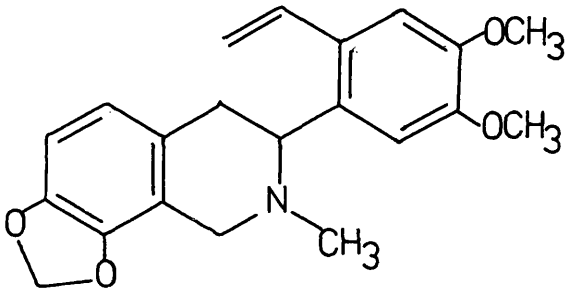
It was hoped to cleave the vinyl group oxidatively to the aldehyde, then to reform the vinyl group by reacting with radiolabelled methyl magnesium bromide followed by dehydration of the alcohol thus formed. Alternatively an organometalic reaction on the acid was to be used.

Reactions were carried out on the vinyl group of the following anhydrocryptopine derivatives:

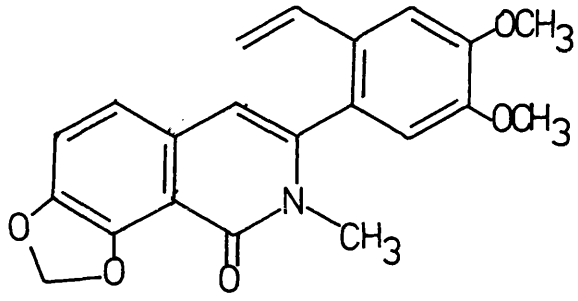
a) 1,2-dihydro-3-arylisoquinoline (170)

b) 1,2,3,4-tetrahydro-3-arylisoquinoline (171)

c) 1,2-dihydro-1,2-oxo-3-arylisoquinoline (172)



(171)



(172)

All three classes were reacted under a variety of oxidising conditions:

i) ozonolysis

ii) OsO_4/potassium periodate

iii) $KMnO_4$/potassium periodate [168]

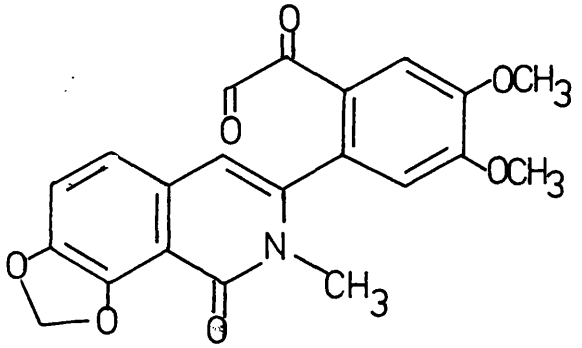
iv) $KMnO_4$/quarternary ammonium halide [169]

No success was achieved although several compounds were detected and assigned structures on the basis of their mass spectra.

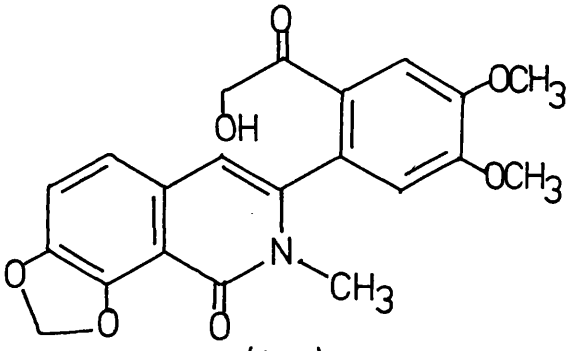
Initial attempts to cleave the vinyl group with potassium permanganate led to the quantitative recovery of the 1,2-dihydro-1,2-oxo- compound. Reaction of this compound with ozone in methylene chloride solution gave no detectable products, and starting material was recovered quantitatively.

In separate experiments the oxo- compound (172) was treated with a permanganate/periodate mixture and with an osmium tetroxide/periodate mixture; in both cases the main component of the recovered material was the starting compound although in both cases an unidentified compound with a molecular weight (M^{+}) of 393 was detected in low concentration in the mass spectrometer.

Reaction of the oxo- compound with potassium permanganate and trimethylcetyl ammonium bromide in benzene ('purple benzene') failed to produce the aldehyde derivative although small quantities of two oxidation products were detected. These have been tentatively assigned the structures (173) and (174) although there was insufficient material for complete characterisation.

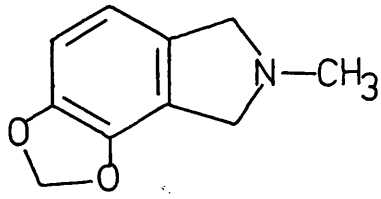


(173)



(174)

Reactions were carried out on anhydrocryptopine in the same way: both permanganate/periodate and osmium tetroxide/periodate oxidations proceeded exothermically and produced tars which proved impossible to separate. It was clear, however, that the desired aldehyde had not been formed. Ozonolysis again proved unsuccessful. Reaction with 'purple benzene' proceeded exothermically, cleaving the molecule to produce the phthalimide (175).



(175)

Reduction of anhydrocryptopine with sodium borohydride in ethanol gave the 1,2,3,4-tetrahydroisoquinoline (171) which on ozonolysis gave the 1,2-dihydro-1 -oxo-isoquinoline in both dichloromethane and in formic acid. Oxidation with both permanganate/periodate and with osmium tetroxide/periodate led to the formation of 1,2,3,4-tetrahydro-1,2-oxo- and 1,2-dihydro-1,2-oxo-isoquinolines in low yield plus unreacted starting material.

Treatment of the tetrahydroisoquinoline with iodine and sodium acetate produced the fully aromatic isoquinolinium iodide.

It is difficult to explain the apparent lack of reactivity of the aryl vinyl group, since this function in other, similar, environments has been shown to cleave. Obviously, in the case of the 1,2-dihydroisoquinoline, and the 1,2,3,4-tetrahydro-isoquinoline the oxidation to the 1,2-oxo- compound is the lower energy reaction path, but this part of the compound, once formed, appears to deactivate the vinyl group although it

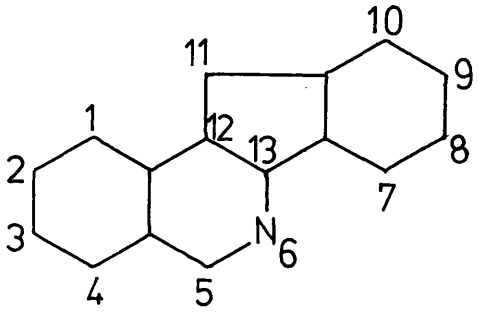
is not in direct conjugation with it.

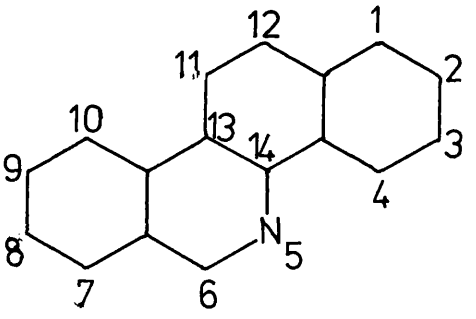
This deactivation could not be overcome by manipulation of conditions or reactants since the energy required to cleave the molecule appears to be less than that required to oxidise the vinyl group.

PART II

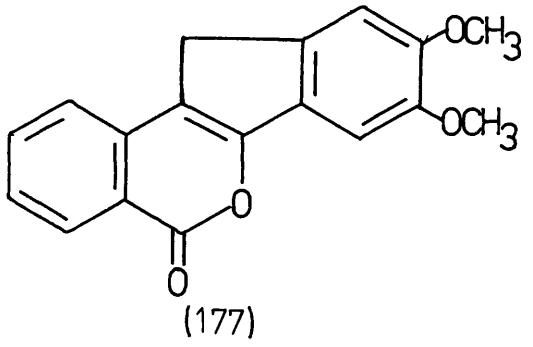
The indeno(1,2-c)isoquinoline route

The indeno(1,2-c)isoquinoline skeleton is numbered in the opposite way to that of the benzo(c)phenanthridines:

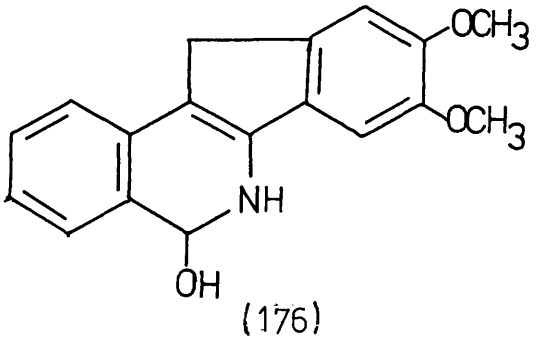




The indeno(1,2-c)isoquinoline system has received little attention until recently. Chatterjea and Mukerjee[170] synthesised (176) by treatment of (177), obtained by polyphosphoric acid cyclisation of an α-benzylhomophthalic acid derivative, with



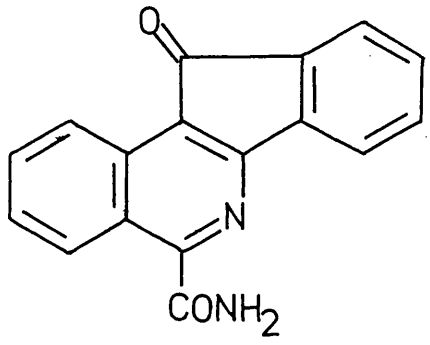
(177)



(176)

ethanolic ammonia.

The basic skeleton (179) has been synthesised by Wawzonek[171] by reductive decarboxylation of the carbamyl derivative (178) formed by reaction of phthaldehyde and sodium cyanide.



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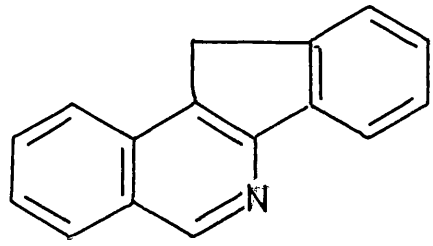
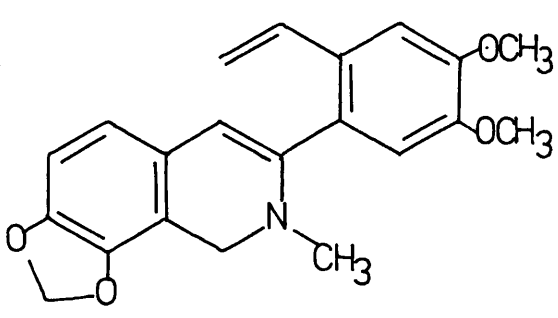
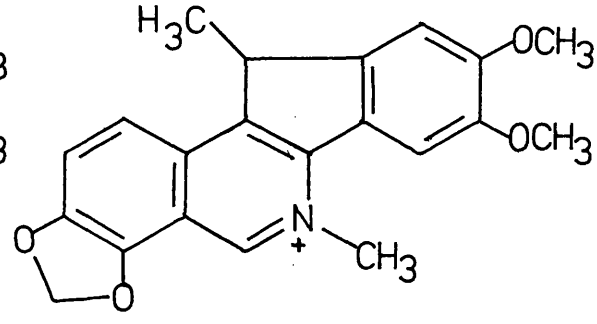


(179)

More recently, in an extensive re-examination of Perkin's work on cryptopine (168)[166], Dyke and Brown[167] showed that the product of the reaction of anhydrocryptopine (180) with concentrated hydrochloric acid was the indeno(1,2-c)isoquinoline epicryptopirubin (181)

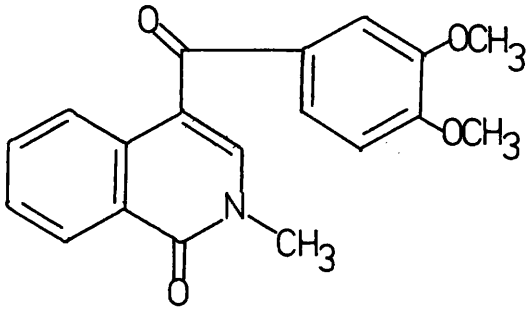


(180)

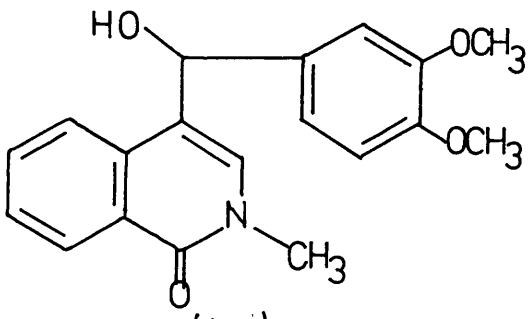


(181)

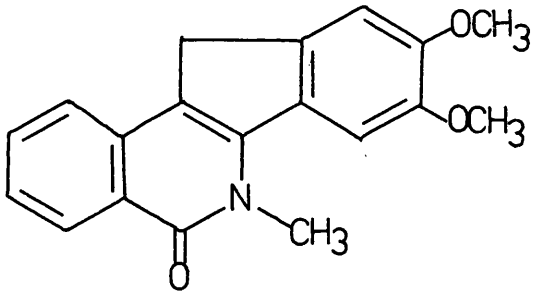
Work by Dyke, Palfreyman et al.[172] showed that sodium borohydride reduction of the 4-acylisoquinoline (182) to the alcohol (183) followed by treatment with ethanolic hydrochloric acid caused ring closure to the indeno(1,2-c)isoquinoline (184), 5-keto-6-methyl-8,9-dimethoxy-11H-indeno(1,2-c)isoquinoline.



(182)



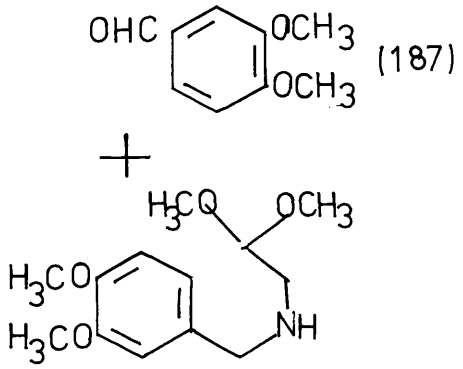
(183)



(184)

The mechanism proposed involves a nucleophillic attack of the isoquinoline C-3 position by the free <u>para</u>- position of the dimethoxybenzyl group, with loss of the benzylic hydroxy function. Rearomatisation of the D-ring leads to the 11H-indeno(1,2-c)isoquinoline.

In a programme of work designed to produce oxygenated analogues of the indeno(1,2-c)isoquinoline ring system Wiggins[74] attempted to form the 4-benzylisoquinolinium salt (185) by acid catalysed condensation of the benzylaminoacetaldehyde-dimethylacetal (186) with veratraldehyde (187).



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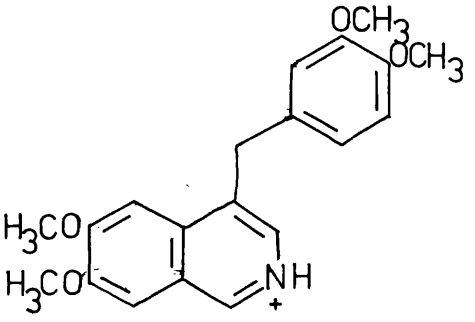
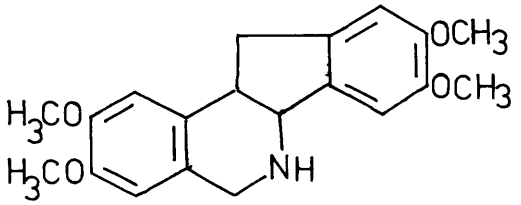
(186)



(185)

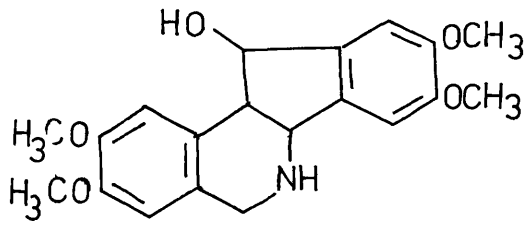
Instead of the 4-benzyl compound the 2,3,8,9-tetramethoxyindeno-(1,2-c)isoquinoline (188) was isolated.



(188)

The mechanism of the reaction is thought to involve nucleophillic attack of the veratraldehyde carbonyl carbon atom by the C-4 position of a proposed 1,2-dihydroisoquinoline intermediate formed by ring closure of the dimethylacetal (186). The intermediate thus formed would ring-close by nucleophillic attack of the isoquinoline C-3 position by the free position <u>para</u>-to the 3-methoxy group of the dimethoxybenzyl moiety to give (189). Dehydration and dehydrogenation would give the product isolated. Variation of reaction conditions optimised the yield, and it was found that the best yields were obtained by heating the reactants at reflux temperature for six hours in 9M hydrochloric acid.

Wiggins then produced the 11-ketoindeno(1,2-c)isoquinoline (190)



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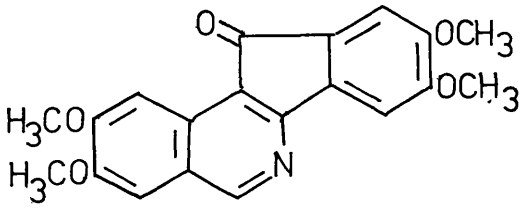
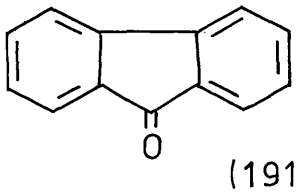


(190)

by treating (188) with sodium dichromate in acetic acid.

It was hoped at this point to mimic the behaviour of fluorenone (191) by ring-expanding the 11-ketoindeno(1,2-c)isoquinoline to the benzo(c)phenanthridine using diazomethane.



(191)

The reaction had been shown to proceed with fluorenone by Schultz,[173] and the infra-red C=O stretching frequencies of the two compounds were close enough (1690cm^{-1} for the 11-ketoindeno-(1,2-c)isoquinoline and 1709cm^{-1} for fluorenone) to expect a similar reaction to proceed with the indeno compound.

Unfortunately, Wiggins was unable to produce the ring expanded product using either etherial solutions of diazomethane or diazomethane generated in-situ. The same lack of reactivity was demonstrated by the methosulphate.

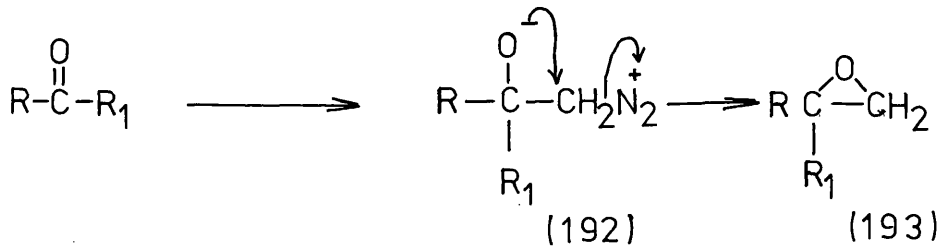
Further investigations by Wiggins showed a complete lack of carbonyl reactivity by the 11-ketoindeno(1,2-c)isoquinoline and by the 11-ketoindeno(1,2-c)isoquinolinium methosulphate although similar ketone reactions had been achieved with fluorenone by Schultz.

This synthetic approach was particularly interesting to this author since ring-expansion of the 11-ketoindeno(1,2-c)isoquinoline with diazo-^{14}C-methane would lead to a benzo(c)phenanthridine labelled at either C-11 or C-12. Choice of suitable precursors and suitable post ring-closure reactions could lead to radiolabelled sanguinarine.

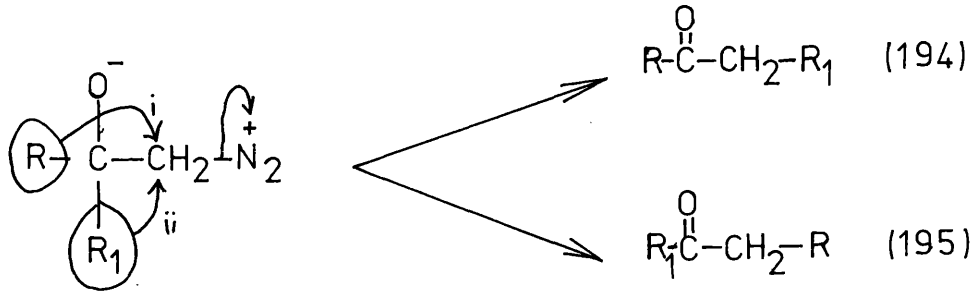
Consequently, and in spite of the unsuccessful attempts described above, it was decided to try this synthetic approach again. It was felt that the similarity of the 11-ketoindeno-compound to fluorenone was such that variation of the conditions of reaction must lead to eventual success.

The reaction of diazomethane with ketones has been reviewed by Gutsche[174] and a mechanism has been proposed. Initial nucleophillic addition of methylene to the carbonyl group forms the zwitterion (192) which decomposes to form either the epoxide (193) or the 1,2- rearrangement products (194) and (195) with loss of molecular nitrogen. All three have been reported but usually

one product is preferred.



(192) (193)

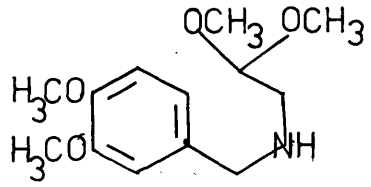


(194) (195)

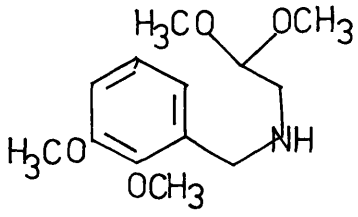
Using the benzylaminoacetaldehydedimethylacetals (196) and (197) in condensation reactions with veratraldehyde this author prepared the 11-ketoindeno(1,2-c)isoquinolines (198) and (199) by the action of sodium dichromate on the respective 11H-indeno(1,2-c)isoquinolines. Ring-expansion of the 11-keto-compounds with etherial solutions of diazomethane[175] failed as before. In-situ production of diazomethane using the powerful diazomethane generator p-tolylsulphonylmethylnitrosamide[176] (200) also failed to produce the desired ring-expansion product.

It was unfortunately found to be necessary to abandon work

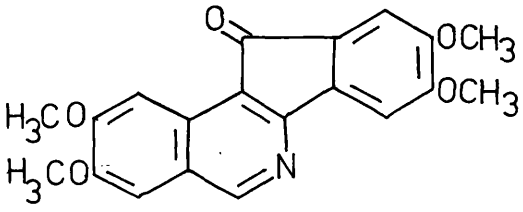
on this project due to lack of time.



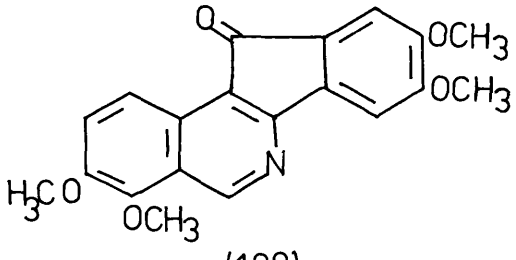
(196)



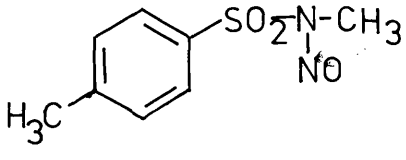
(197)



(198)

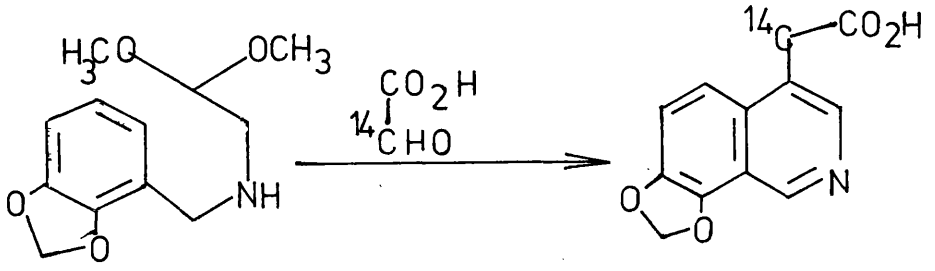


(199)

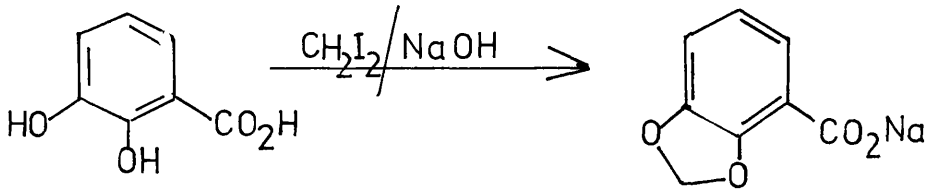


(200)

Although both routes studied above were interesting and deserve further study it is considered that the best and simplest method of inserting a label at C-11 would be to use the method of Dyke et al mentioned in Chapter 1. ^{14}C would be introduced as 1-^{14}C-glyoxylic acid :-

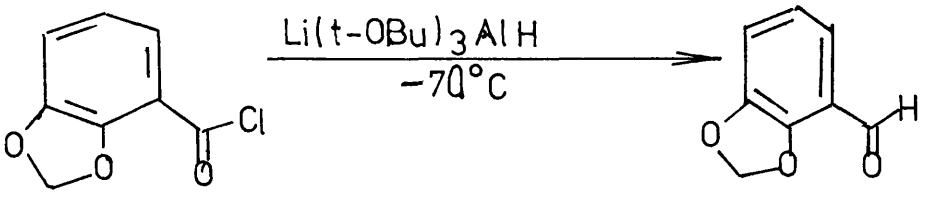


Some development work was done on this synthetic route, mainly aimed at improving the yields of the early stages. To this end, greater homogenisation of methylene iodide led to increased yields and shorter reaction times for the production of the methylenedioxy acid (201).



(201)

Similarly the Rosenmund catalytic reduction of the acid chloride to the aldehyde (202) was replaced by reduction of the acid chloride by an aluminium hydride reduction which led to a more reproduceable reaction in terms of yields and products.



(202)

EXPERIMENTAL

PART I

Preparation of isocryptopine chloride (169)

To cryptopine (168) (13.9gm,0.036M) was added phosphorus oxychloride (28ml). The buff coloured slurry thus formed was heated at reflux temperature for 0.75 hours, after which time it was poured into cold water, with stirring and cooling. The solid, which was collected by filtration, was recrystallised from 2M hydrochloric acid to give isocryptopine chloride (11.5gm,85%) ν_{max} 1610cm^{-1}.

Preparation of anhydrocryptopine (170)

Isocryptopine chloride (1.70gm,4.5mM) was heated with a 25% solution of potassium hydroxide in methanol at 100°C for 0.75 hours. The reaction mixture was cooled and the solids collected by filtration. After washing with water the solid material was recrystallised from methanol to give anhydrocryptopine (1.0gm,60%) MPt 104°-108°C
ν_{max} 1610cm^{-1} 1605cm^{-1}.

Attempted osmolysis of anhydrocryptopine

Anhydrocryptopine (480mg,1.3mM) was dissolved in ($LiAlH_4$ distilled) dioxane (15ml) and water (4ml). Addition of osmium tetroxide (5mg) produced an immediate browning. Addition of sodium periodate (500mg) caused an exothermic reaction. After 2.0 hours the mixture was diluted with water (50ml) and extracted with chloroform (4 x 50ml);

The combined organic extracts were washed with water (2 x 50ml) and dried over magnesium sulphate. Removal of chloroform under reduced pressure afforded a black tar which proved unseparable.

Attempted permanganate/periodate oxidation of anhydrocryptopine

Anhydrocryptopine (500mg,1.35mM) was dissolved in the minimum amount of acetone and 20ml of 0.05M potassium carbonate solution added as a buffer. Addition of 0.2M sodium periodate solution (10ml) followed by 0.0005M potassium permanganate solution caused immediate reaction with generation of heat. After 2.0 hours the reaction mixture was treated with sodium thiosulphate solution and extracted with chloroform (3 x 50ml). After washing with water (2 x 50ml) and drying over magnesium sulphate the chloroform was removed to yield an intractable tar.

Attempted ozonolysis of anhydrocryptopine

Anhydrocryptopine (110mg,0.3mM) was dissolved in hot methanol (25ml) and rapidly cooled to produce a fine suspension. A mixture of ozone and oxygen from an ozone generator was bubbled through the suspension for three hours, when zinc dust (50mg) and water (25ml) were added to destroy the ozonide. The mixture was allowed to stand for twelve hours after which time the reaction mixture was worked-up. Starting material was recovered quantitatively.

Preparation of dihydroanhydrocryptopine (171)

Anhydrocryptopine (2.0gm,5.4mM) was stirred in 50% aqueous ethanol (100ml) and sodium borohydride (160mg) added in portions. After overnight stirring the reaction mixture was concentrated under reduced pressure. Water (40ml) was added and the suspension extracted with chloroform (3 x 50ml). The combined organic extracts were washed with water (2 x 50ml), dried over magnesium sulphate, and the chloroform removed under reduced pressure to give the tetrahydro- compound (171) as a rapidly deteriorating brown semisolid (1.80gm,90%).

ν_{max}=1610cm^{-1}, M^+ 353.

The product was isolated as the perchlorate salt and recrystallised from aqueous ethanol.

Reoxidation of dihydroanhydrocryptopine

Dihydroanhydrocryptopine (500mg,1.3mM) was treated with 5% aqueous potassium carbonate solution (30ml) and the solution extracted with diethyl ether (3 x 50ml). The combined organic extracts were washed with water (2 x 50ml), dried over magnesium sulphate and the diethyl ether removed under reduced pressure. The residue was treated with iodine (1gm,7.8mM) in a solution of ethanol (100ml) and sodium acetate (5gm). After stirring for 1.0 hours the reaction was treated with a solution of sodium thiosulphate (2.0gm) in water (100ml) and stirred for a further 0.5 hours. Work-up produced a brown glass which was triturated with petroleum ether (40°-60°) to give a pale brown solid (380mg). Mass spectrometry showed this

to be a mixture of the fully aromatic isoquinolinium iodide and ketoanhydrocryptopine. The solid was taken up in 2M hydrochloric acid and filtered. Addition of sodium perchlorate gave the fully aromatic isoquinolinium perchlorate as a yellow solid. MPt 280°C-282°C. M^+350.

Osmolysis of dihydroanhydrocryptopine

Dihydroanhydrocryptopine (500mg,1.3mM) was dissolved in dioxane (15ml) and water (4ml). Osmium tetroxide (5mg) was added, followed by the stepwise addition of solid sodium periodate (500mg) over 0.5 hours, after which the reaction was stirred for a further two hours. Water (40ml) was added and the solution worked-up. The semisolid isolated (450mg) was a mixture of starting material (M^+353), and the 1-oxo-derivatives of anhydrocryptopine (M^+365) and dihydroanhydrocryptopine (M^+367) by comparative tlc.

Ozonolysis of dihydroanhydrocryptopine

Dihydroanhydrocryptopine (1gm,2.7mM) was dissolved in 60% formic acid (50ml) and a mixture of ozone and oxygen bubbled through the solution for 3.0 hours. The solution was neutralised with 30% sodium hydroxide solution and treated with zinc dust. Filtration was followed by extraction of the filtrate with dichloromethane (3 x 50ml). The combined organic layers were washed with water (2 x 50ml) and dried. Removal of the dichloromethane afforded ketoanhydrocryptopine (0.25gm) ν_{max}1662cm^{-1},M^+365. Basification of the aqueous filtrate above with 30% sodium hydroxide solution was followed by extraction

with diethyl ether (3 x 50ml) which after drying and evaporation under reduced pressure gave the starting material (0.60gm).

Periodate/permanganate oxidation of dihydroanhydrocryptopine

Dihydroanhydrocryptopine (1.0gm,2.7mM) was dissolved in dioxane (25ml) and treated with a solution of sodium periodate (0.75gm) and potassium permanganate (25mg) in water (5ml). After stirring for 2.0 hours the solution was diluted with water and extracted with chloroform (3 x 50ml) which on evaporation gave an oil (0.6gm) which proved to be a mixture of starting material and ketoanhydrocryptopine.

Preparation of ketoanhydrocryptopine (172)

Anhydrocryptopine (3.0gm,8.1mM) and active manganese dioxide (2.0gm) were stirred in dry benzene overnight. After evaporation of benzene the solid was extracted with hot glacial acetic acid which was poured into water and the aqueous solution extracted with diethyl ether (4 x 100ml). The combined organic extracts were washed with 5% sodium bicarbonate solution (2 x 100ml), water (2 x 100ml) and dried over magnesium sulphate. Evaporation of solvent under reduced pressure gave the required isocarbostyril (2.1gm,72%) ν_{max}1662cm^{-1}.

Ozonolysis of ketoanhydrocryptopine

Ketoanhydrocryptopine (0.5gm,1.3mM) was dissolved in dichloromethane (50ml) and a mixture of ozone and oxygen bubbled through for three hours. The solution was then shaken with zinc powder (200mg) in glacial acetic acid (30ml) for 1.0 hours. Zinc was removed by filtration and water (50ml) added. The layers were separated and the organic phase retained. The aqueous phase was extracted with dichloromethane (3 x 50ml) and the combined organic layers washed with aliquots of water until the washings were neutral. Treatment of the organic phase in the normal way produced the starting material (0.45gm) ν_{max}1662cm^{-1}.

Reaction of ketoanhydrocryptopine with potassium permanganate solublised in benzene ('Purple benzene')

To Potassium permanganate (1gm) in water (10ml) was stirred rapidly for ten minutes. The solution was cooled and benzene (10ml)

and cetyltrimethylammonium bromide (0.1gm) added, the benzene layer immediately took on a purple colouration. Ketoanhydrocryptopine (0.25g,0.7mM) in benzene (10ml) was added and the two phase mixture stirred for 2.0 hours, after which time saturated sodium thiosulphate solution was added to discharge the colour. After acidification, the layers were separated and the aqueous layer extracted with benzene (2 x 20ml) and the combined organic extracts washed with water (2 x 20ml) and dried over sodium sulphate. Removal of benzene under reduced pressure left a solid (50mg), which recrystallised from petroleum ether (40-60°C) as yellow plates. No definite melting point could be obtained and low energy (11eV) mass spectrometry showed the solid to be a mixture. M^+397 (100%), M^+395 (90%). ν_{max} 1710cm^{-1}.

Attempted osmoylysis of ketoanhydrocryptopine

Ketoanhydrocryptopine was reacted with osmium tetroxide and sodium periodate in a manner similar to that described above. Starting material was recovered quantitatively.

Attempted oxidation of ketoanhydrocryptopine with permanganate/periodate

Ketoanhydrocryptopine was treated with potassium permanganate and sodium periodate in a manner similar to that described above. Starting material was recovered quantitatively.

PART II

Preparation of 2,3,8,9-tetramethoxy-11H-indeno(1,2-c)isoquinoline( )

3,4-dimethoxybenzylaminoacetaldehydedimethylacetal (17gm, 0.067M) was heated at reflux temperature with 3,4-dimethoxybenzaldehyde (12gm, 0.072M) for 6.0 hours in 9M hydrochloric acid (100ml). The reaction mixture was cooled and extracted with diethyl ether (2 x 100ml). The aqueous solution was basified with dilute ammonia and extracted with chloroform (3 x 150ml); the combined extracts were washed with water, dried over magnesium sulphate and the solvent evaporated to yield an oil. Dissolution of the oil in acetone followed by treatment with dry hydrogen chloride produced the hydrochloride salt (4.1gms) which was recrystallised from pyridine (3.5gms, 16%). MPt 268-270°C; ν_{max} 1620cm^{-1}, 1580cm^{-1}
nmr (TFA): 14 proton complex 4.1-4.35ppm (4 x OCH_3, C-11 methylene);
4 aromatic protons (singlets) 7.40, 7.50, 7.60, 7.85ppm;
1 proton (multiplet) 9.2ppm.
M^+ 338.

Preparation of 11-keto-2,3,8,9-tetramethoxyindeno(1,2-c)-isoquinoline (198)

A solution of 2,3,8,9-tetramethoxyindeno(1,2-c)isoquinoline (1.0gm, 3mM) in glacial acetic acid (150ml) was treated with a solution of sodium dichromate (1.0gm) in glacial acetic acid (100ml). After heating for 3.0 hours at 100°C the glacial acetic acid was removed under reduced pressure to yield a solid that was dissolved in water (200ml) and extracted with chloroform, (3 x 150ml).

After washing and drying the chloroform was removed to yield a red semisolid which was eluted down an alumina column with benzene/chloroform (9:1). After removal of benzene/chloroform the red residue was recrystallised twice from benzene to give a crystalline solid (0.39gm,40%). MPt 182-185°C

ν_{max} 1690cm^{-1}, 1580cm^{-1}

nmr(TFA) 12 proton complex 3.0-3.3ppm (4 x OCH_3); 3 proton complex 7.45-7.55ppm (3 x aromatic H); 1 proton singlet 8.2ppm (1 x aromatic H); 1 proton singlet 9.1ppm (1 x aromatic H)

M^+351.

Attempted ring-expansion

a) External generation of diazomethane.

To a solution of 11-keto-2,3,8,9-tetramethoxyindeno (1,2-c)-isoquinoline (0.6gm,1.7mM) in methanol (100ml) was added an excess of an ethereal solution of diazomethane. After standing at room temperature for several hours the solvent was removed under reduced pressure.

Starting material was recovered.

b) In-situ generation of diazomethane

11-keto-2,3,8,9-tetramethoxyindeno(1,2-c)isoquinoline (40mg,0.11mM) was dissolved in distilled chloroform (30ml) and methanolic potassium hydroxide solution (20%,20ml) and water (20ml) added. To this was added N-methyl-N-nitroso-p-tolylsulphonamide (200)(1gm) and freshly precipitated silver oxide (100mg). After stirring at 40°C for 4.0 hours the mixture was allowed to separate; the chloroform layer

was collected and the aqueous phase extracted with further chloroform (2 x 30ml). The combined chloroform extracts were washed with water (2 x 20ml), dried over magnesium sulphate and the chloroform removed under reduced pressure to give returned starting material (30mg,75%).
No other material was isolated.

Preparation of 2,3,7,8-tetramethoxyindeno(1,2-c)isoquinoline ( )

2,3-dimethyoxybenzylaminoacetaldehydedimethylacetal (10gm,0.039M) and 3,4-dimethoxybenzaldehyde (11gm,0.066M) were heated in 9M hydrochloric acid (45ml) for 6.0 hours at 100°C. The reaction mixture was cooled and extracted with diethyl ether (2 x 50ml). The red solution was basified with dilute ammonia and extracted with chloroform (3 x 150ml). The combined chloroform extracts were washed with water (2 x 100ml), dried over magnesium sulphate and evaporated to give an oil which was taken up in acetone and treated with dry hydrogen chloride. After two hours a bright yellow crystalline product was collected, washed with ethanol and recrystallised from pyridine (1.3gms,10%) MPt 240-242°C ν_{max} 1620cm^{-1}, 1580cm^{-1} nmr 14 proton complex 3.9-4.2ppm, (4 x OCH_3, C-11 methylene); 1 proton singlet 7.1, 2 proton singlet 7.9, 1 proton singlet 8.2, 1 proton singlet 9.2ppm.

Preparation of 11-keto-2,3,7,8-tetramethoxyindeno(1,2-c)-isoquinoline (199)

2,3,7,8-tetramethoxyindeno(1,2-c)isoquinoline (337mg,1mM) was dissolved in glacial acetic acid (150ml) and treated with a solution of sodium dichromate (600mg) in glacial acetic acid (150ml). After heating at 100°C for 3.0 hours the glacial acetic acid was removed under reduced pressure to leave a solid that was dissolved in water (100ml) and extracted with chloroform (3 x 50ml). After washing with water (2 x 50ml) and drying with magnesium sulphate the chloroform was removed to give a red brown solid (150mg) which was eluted down an alumina column with benzene/chloroform (9:1). Removal of benzene/chloroform left a red glass which was induced to crystallise with methanol (100mg,30%) MPt 210-215°C

ν_{max} 1690cm^{-1}, 1580cm^{-1}

nmr 12 proton complex 4.1-4.2ppm (4 x OCH_3); 1 proton singlet, 6.8ppm, 1 proton singlet 7.6ppm, 2 proton singlet 7.8ppm, 1 proton singlet 9.3ppm.

Attempted ring-expansion of 11-keto-2,3,7,8-dimethoxyindeno-(1,2-c)isoquinoline

In-situ generation of diazomethane by alkaline decomposition of N-methyl-N-nitroso-p-tolylsulphonamide was carried out using the conditions described above.

The starting material was recovered.

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